

FITNESS EFFECTS OF KINSHIP DEPEND ON ECOLOGICAL CONTEXT IN THE
AMERICAN TOAD (*ANAXYRUS AMERICANUS*)

By

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ABSTRACT

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Studies of cooperation often ask how variation in kinship impacts the inclusive fitness payoffs of altruism. Hamilton's rule defines this as a function of cooperation's costs and benefits, which in principle can vary widely across ecological contexts. In this dissertation, I address how kinship influences fitness, how selection balances fitness costs and benefits, and how the effects of kinship modulate the effects of other aspects of an organism's environment using the American toad system.

I first asked whether tadpoles use chemical cues to perceive differences in relatedness and whether this variation affects the response of several fitness proxies to environmental cues. I found that tadpole growth rates differed in response to cues of resource and kinship environment. In another experiment, growth rate differed based on cues of relative size, with larger tadpoles outperforming smaller partners. This was affected by kinship, at least for smaller tadpoles, who grew more rapidly with a sibling. This indicates that chemical cues communicate information necessary for tadpoles to perceive aspects of their environment, which interact with relatedness to affect fitness.

I then investigated whether relatedness influences growth and development in experimental groups of tadpoles, and whether other factors – such as density and nutrient availability – impact the fitness benefits of grouping with kin. In our experiments, groups composed of full-sib kin reached metamorphosis faster and at a larger size than mixtures of different sib groups. The benefits of these fitness components were

significant in more competitive, resource-scarce environments, but negligible in less-competitive, resource-abundant environments. Kinship and resource abundance have an interactive effect on the fitness components we measured.

Finally, I assessed tadpole aggregation preferences for kin compared to non-kin in the presence and absence of predator cues. In the presence of predator cues, tadpoles may be more likely to choose kin over non-kin. While increased body mass might result in a tadpole being more likely to avoid conspecifics in the presence of predator cues, we saw that larger tadpoles potentially increased the probability of choosing kin over non-kin. While these results were not significant, indicating that predator avoidance is likely not the primary driver of kin aggregation behavior in this species, they are suggestive of a kin-selected benefit to grouping.

Taken together, these results help us understand the contexts in which we might expect relatedness to affect fitness, which could further contribute to our understanding of the evolution of social behavior. This emphasizes that the fitness benefits of kin-directed behavior are not identical in all circumstances, and that the ratio of costs to benefits may drive the evolution of different strategies depending on the environment.

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INTRODUCTION

Kin can be an organism's most intense competitors or its most reliable allies, depending on the circumstances. The development of kin selection theory and the concept of inclusive fitness provided a theoretical framework to help us understand how variation in genetic similarity among individuals may lead to differences in the strength of competition and cooperation (Gardner and West 2014). Hamilton's rule (Hamilton 1964) provides a simple rule-of-thumb for finding the evolutionary optimum between selfishness and cooperation among related individuals. This specifies that a gene for some altruistic behavior will spread if the average relatedness between the individual performing an action and the recipient of that action multiplied by the benefit gained by the recipient due to that action is greater than the cost to the actor of performing that action. This concept has provided the basis for a great deal of sibling conflict theory, as it provides not only the conditions necessary for altruism but also, if reversed, the conditions under which selfishness will prevail.

This last point is critical, as Hamilton's rule highlights that it is not just relatedness that matters in determining whether an action is favored by selection, but also the ratio of fitness costs and benefits. A great deal of focus has been placed on how relatedness of interacting individuals affects the evolution of cooperation, with less consideration being given to how the context in which these interactions take place can alter the costs and benefits of cooperation, which is likely a significant part of the story (Bourke 2014). Rather than focusing simply on cases where there are dramatic fitness costs to the actor, theoretical work has also provided a way to think about more subtle costs of cooperation. The optimal amount of some resource to take depends on the marginal

fitness benefits an individual will receive from taking in more of the resource (Parker 1989), which could have implications for the cooperation-competition balance.

Individuals should be expected to vary the intensity of resource competition according to relatedness. In some cases, smaller siblings will increase inclusive fitness by deferring to larger, healthier kin, while in other cases larger siblings increase inclusive fitness by allowing smaller kin primary access to food (Parker et al. 1989, Yamamura and Higashi 1992). Kin interactions in line with these predictions have been seen in a variety of taxa (Willson et al. 1987, Tonsor 1989, Briskie et al. 1994, Johnstone and Roulin 2003, Boncoraglio et al. 2009, Madden et al. 2009, File et al. 2011), and we expect them to prove adaptive in amphibians as well. This provides us with novel opportunities to investigate how variation in the environment influences the effects of kinship on development and fitness.

Some amphibian species form aggregations in the larval stage, often composed of siblings (Waldman and Adler 1979, Waldman 1982, Waldman 1986, Pfennig et al. 1993). These provide many benefits, such as anti-predator defense and social foraging (Watt et al. 1997, Sontag et al. 2006), but the dynamics become more complicated with limited resources and increased potential for competition. A variety of population responses have been seen when tadpoles interact at high densities (Steinwascher 1979, Breden and Kelly 1982, Semlitsch and Caldwell 1982, Travis and Trexler 1986, Warner et al. 1991, Newman 1994), but one common trend is for larger tadpoles to inhibit the growth of smaller conspecifics when competing for limited resources. This presumably involves the use of a waterborne chemical agent and not just increased aggression, as we see these inhibitory effects even when tadpoles are only raised in

water from crowded conditions (Light 1967, Steinwascher 1978, Griffiths et al. 1993). There is evidence that chemical cues are used to recognize kin and influence behavior in some kin-aggregating species (Waldman 1985, Pfennig and Frankino 1997), and some cues seem to provide information about size (Lee and Waldman 2002), so this may provide a mechanism for communicating growth information to siblings. Although larger tadpoles may simply suppress growth in their competitors, the degree of relatedness between conspecifics has the possibility to alter the response. Relatedness has been considered in some experiments on growth and development (Hokit and Blaustein 1994, Hokit and Blaustein 1997, Saidapur and Girish 2001, Pakkasmaa and Aikio 2003, Gramapurohit et al. 2008), and the performance of smaller tadpoles is sometimes improved by grouping with kin (Jasienski 1988, Smith 1990). Overall, mixed results have been found and more work is necessary to understand how kin recognition might interact with group density, growth suppression, and environmental context to influence fitness.

The American toad (*Anaxyrus americanus*) has been shown to preferentially aggregate with kin during the larval stage in both lab and field experiments (Waldman and Adler 1979, Waldman 1982). This preference is seen even when tadpoles are only exposed to water from related and unrelated individuals (Waldman 1986) and disappears when olfaction is blocked, suggesting a waterborne chemical cue is involved (Waldman 1985). There is no evidence of active cannibalism in this species (Heinen and Abdella 2005), suggesting there must be other less-obvious benefits to kin recognition. Toads are also explosive breeders that may reproduce in a variety of water bodies ranging in size and permanence, leading to significant natural variation in

relatedness, density, and resource availability within these breeding ponds. This makes them a good system for testing questions of how relatedness influences fitness within different contexts and whether chemical cues might mediate these effects.

I am broadly interested in how kinship influences fitness, how selection balances fitness costs and benefits, and how the effects of kinship modulate the effects of other aspects of an organism's physical and social environment. In this dissertation, I will address several of these questions using the American toad system. In Chapter One, I ask whether tadpoles use chemical cues to perceive differences in relatedness and whether variation in relatedness affects the response of several fitness proxies to environmental cues. In Chapter Two, I investigate whether relatedness influences growth and development in experimental groups of tadpoles, and whether other factors – such as density and nutrient availability – impact the fitness benefits of grouping with kin. Finally, in Chapter Three, I assess tadpole aggregation preferences for kin compared to non-kin in the presence and absence of predator cues. Taken together, these results help us understand the contexts in which we might expect relatedness to affect fitness, which could further contribute to our understanding of the evolution of social behavior.

CHAPTER ONE:
***CHEMICAL CUES OF RELATEDNESS MODIFY THE EFFECTS OF
RESOURCE AVAILABILITY AND RELATIVE SIZE ON FITNESS INDICES
IN THE AMERICAN TOAD (ANAXYRUS AMERICANUS)***

Introduction

Animals extract information from their physical and social environment in many ways to make behavioral decisions. Many taxa rely on chemical cues and signals to communicate information relevant to fitness (Steiger et al. 2011). Chemical communication is used to locate mates or appropriate sites for reproduction (Buchinger et al. 2015), evaluate reproductive status or mate quality (Johansson and Jones 2007), warn conspecifics of predator risk (Schoeppner and Relyea 2009), and signify individual or group identity (Wyatt 2010). One aspect of identity that may impact social behavior is relatedness, as individuals may behave differently in the presence of kin versus non-kin.

The ability to distinguish kin from non-kin is essential for kin-directed behavior (Penn and Frommen 2010), which can be crucial for increasing inclusive fitness. This idea holds that an individual's fitness can be increased by benefitting relatives who share genes by common descent in addition to their own direct fitness (Hamilton 1964, Gardner and West 2014). Theory predicts that, in some cases, smaller siblings will increase inclusive fitness by deferring to larger, healthier kin, while in other cases larger siblings increase inclusive fitness by allowing smaller kin primary access to food (Parker et al. 1989, Yamamura and Higashi 1992). This pattern has been seen in several taxa where individuals may not be able to avoid interacting with relatives (Johnstone and Roulin 2003, Bulmer et al. 2007, Madden et al. 2009, File et al. 2011) and might prove

adaptive in amphibians as well, where some species are similarly dispersal-limited at the larval stage.

Optimizing inclusive fitness in the manner described above requires reliable information about the condition, as well as the relatedness, of nearby individuals, and amphibians are a system well-suited to communicate this. There is evidence that chemical cues are used to recognize kin and influence behavior in some kin-aggregating species (Waldman 1985, Pfennig and Frankino 1997). Some chemical cues also provide information about size (Lee and Waldman 2002), so this may provide a mechanism for communicating size information to siblings. Mass at metamorphosis is a commonly used proxy for fitness in larval amphibians (Earl and Whiteman 2015), and larger size can increase survival once toads enter the terrestrial environment (Sams and Boone 2010). Conspecific size may also provide information about whether individuals are likely to be related and whether consuming them provides an inclusive fitness benefit in species exhibiting cannibalism (Crossland et al. 2011, Dugas et al. 2016). If tadpoles vary the intensity or manner of competition based on competitor identity, body size will likely influence the fitness costs and benefits of competition.

Another factor that should alter the intensity of competition is resource availability. Collins (1979) suggests that when resources are more limited but consistently present, we expect to see early-metamorphosing tadpoles emerge at the minimum size necessary for metamorphosis, in order to escape competition in the aquatic environment. Those that metamorphose later can reach a larger size given less competition for the consistent resource pool. When resources are more plentiful, all tadpoles should metamorphose at larger sizes, showing no relationship between size at

metamorphosis and time to metamorphosis (Collins 1979). Being able to perceive the resource potential of the environment should allow tadpoles to shift their development in a way that maximizes fitness, which may vary depending on the relatedness and size of competitors.

Some evidence suggests that larger tadpoles can suppress the growth of their competitors (Light 1967, Steinwascher 1978, Griffiths et al. 1993), but this pattern may be affected by relatedness between individuals. In some experiments, the smallest tadpoles in a group have been larger at metamorphosis when grouped with kin versus non-kin (Jasienski 1988, Smith 1990). Here we propose that larger individuals aggregating with kin may be able to avoid producing a signal of growth suppression rather than suppressing all conspecifics, or they may produce a signal that affects kin behavior in other ways. Individuals may change their foraging behavior when in the presence of kin or reduce the amount of time spent on interference or exploitation competition. It is important to understand how the relatedness of a group might influence the behaviors and fitness strategies we see, and which factors affect the costs and benefits of these strategies. This would help us to understand how these different contexts influence fitness, allowing us to better predict how individuals are likely to respond given their circumstances.

The American toad (*Anaxyrus americanus*) has been shown to preferentially aggregate with kin during the larval stage in both lab and field experiments (Waldman and Adler 1979, Waldman 1982). This preference is seen even when tadpoles are only exposed to water from related and unrelated individuals (Waldman 1986) and disappears when olfaction is blocked, suggesting a waterborne chemical cue is involved

(Waldman 1985). There is no evidence of active cannibalism in this species (Heinen and Abdella 2005), suggesting there must be other less-obvious benefits to kin recognition. American toads are also explosive breeders that may reproduce in a variety of water bodies ranging in size and permanence, leading to significant natural variation in relatedness, density, and resource availability within these breeding ponds. This makes them a good system for testing questions of how relatedness influences fitness within different contexts and whether chemical cues might mediate these effects.

We devised a set of experiments to determine whether differences in relatedness would be perceived by tadpoles and result in differences in fitness indices. We also inquired whether other aspects of the tadpole's physical and social environment would influence this relationship. We first considered the impacts of differences in kinship on growth and survival when perceived resource availability to conspecifics in the shared water source differed between treatments. Our second experiment considered whether a tadpole's relative size influenced the effect of kinship on growth and survival. In both cases, focal tadpoles received water from these different treatments, but treatment groups did not vary in the containers in which they were housed or the amount of food they received.

Table 1.1. Hypotheses for the underlying relationship between tadpole fitness indices and chemical cues in the water.

H0: Chemical cues do not impact tadpole growth and survival
H1: Tadpoles use chemical cues to increase direct fitness
Prediction 1: Tadpoles grow less rapidly with reduced resource availability to conspecifics across treatments regardless of kinship
Prediction 2: Smaller tadpoles grow less rapidly regardless of kinship
H2: Tadpoles use chemical cues to increase inclusive fitness
Prediction 1: Tadpole growth with reduced resource availability to conspecifics varies with kinship treatment
Prediction 2: Smaller tadpoles grow more rapidly when partnered with kin

We considered several possibilities about the underlying influence of waterborne chemical cues on tadpole fitness (Table 1.1). If tadpoles are not able to perceive differences in the kinship and growth rate of conspecifics based on water alone, we should not expect to see any differences in growth rate between treatments. If chemical cues in water do impact tadpole fitness, they could simply result in improved performance by tadpoles who perceive themselves to be in a superior environment with no effect of relatedness, or information about body condition and food availability in transferred water could interact with relatedness to lead to differences in growth and survival in the presence of kin cues. By manipulating relatedness as well as other factors that could influence inclusive fitness, we hoped to determine how the interactions between them work to increase tadpole fitness.

Methods

Experiment 1: Relatedness and Food Levels

To test our first prediction for each of hypotheses 1 and 2 (Table 1.1), we set up an experiment in which we exposed individual focal tadpoles to water from different source tanks. These water source tanks consisted of different tadpole clutches receiving food at either abundant or scarce levels, while all focal tadpoles received resources at the abundant level. This allowed us to test whether potential chemical cues in the water could affect focal tadpole growth and development, resulting in different patterns based on perceived group relatedness and resource availability.

We collected amplexed pairs of adult American toads from breeding choruses in several locations in southwest Michigan in April 2012. These locations included the

Experimental Pond Laboratory and Lux Arbor Reserve at the Kellogg Biological Station (KBS). Pairs were isolated in buckets between 24 and 48 hours and given a chance to lay eggs, at which point we returned them to their capture site. Four pairs laid clutches for use in this experiment. Each clutch was split into multiple single-family aquarium tanks until needed.

A subset of tadpoles was taken from each clutch and evenly split into two 20-gallon aquaria of 300 tadpoles each. These tanks corresponded to resource level treatments within the experiment, based on Glennemeier and Denver's food levels required for "normal growth" compared to "inducing competition" (Glennemeier and Denver 2002). Our abundant resource tanks received three pellets of Zoo Med aquatic frog and tadpole food per tadpole, consistent with Glennemeier and Denver's food levels for "normal growth," and the scarce resource tanks received one pellet per tadpole, a level that would be likely to induce competition.

We then set up 240 focal tadpoles for the experiment, taken from the same clutch-specific tanks used to populate the treatment aquaria. Each tadpole was placed within an individual container containing approximately 465 mL of water from one of the aquarium tanks, depending on to which treatment the tadpole was assigned. Tadpoles received water from a tank containing either kin (same clutch) or non-kin (different clutch) receiving abundant resources (three pellets per tadpole per day) or scarce resources (one pellet per tadpole per day) (Fig. 1.1). All focal tadpoles received three pellets per day. Other than differing potential cues in water, the conditions were identical for all 240 focal tadpoles. We recorded each focal tadpole's mass, snout-vent length (SVL) and Gosner stage (Gosner 1960) and placed it in the appropriate container.

Containers were set up in blocks of 120 on May 26 and 27. By this point tadpoles had reached developmental stage 25 (Gosner 1960), when independent feeding begins. The date a focal tadpole was added to its container was considered its start date for measuring the larval period. Sixty tadpoles were assigned to each treatment – receiving water from kin who were given abundant resources, kin who were given scarce resources, non-kin who were given abundant resources, or non-kin who were given scarce resources.

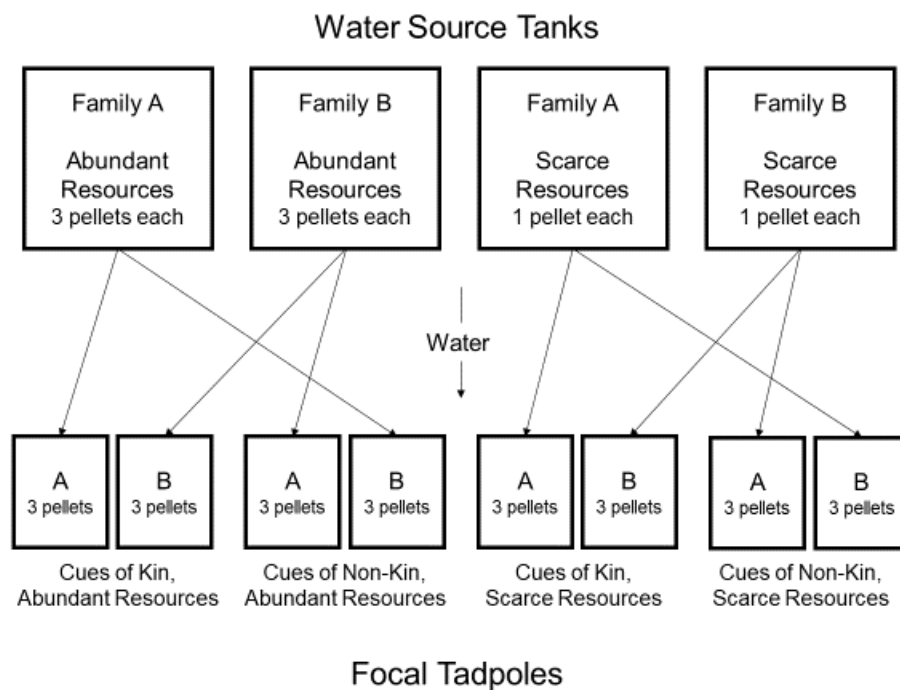


Figure 1.1. A schematic of the experimental design testing how focal tadpoles respond to cues of relatedness and food availability. Water source tanks contain tadpoles from particular families receiving abundant resources (three pellets per tadpole) or scarce resources (one pellet) each day. Focal tadpoles are housed in individual containers where they receive three pellets a day. Focal tadpole containers received water from one of the source tanks depending on their assigned treatment.

Focal and aquarium tank tadpoles were given their assigned food daily. Focal tadpoles' mass, SVL, and Gosner stage were recorded weekly. We also changed the water in the focal containers during the weekly measurement. Tadpoles were removed

from the experiment once they had reached metamorphosis, defined as Gosner stage 42 (at least one forelimb visible), or when they died, whichever came first. After 54 days, 112 tadpoles reached metamorphosis, while 128 did not survive to metamorphosis. While some of this mortality may be the result of smaller tadpoles reacting poorly to being handled, such individuals are also likely to face increased mortality in natural populations (Heinen and Abdella 2005); while high, these mortality levels are likely within the range of variation tadpoles may experience depending on their pond.

Statistical Analysis

After determining that there was no significant nonlinearity in the relationship between mass and date for the focal tadpoles, we found each tadpole's growth rate over the course of the experiment by regressing tadpole mass on date and taking the slope. We used the *lme4* package (Bates et al. 2015) in R (R Core Team 2017) to test whether kinship and resource cues in water affected tadpole growth rate. To do this, we fitted our response variable of tadpole growth rate to fixed effects of family, kinship cue (kin or non-kin), resource cue (abundant or scarce resources), and whether the individual ultimately reached metamorphosis, as well as interactions between these main effects. We also included the water source tank and the source tank of each focal tadpole as random intercepts in the models, to account for differences among tanks that might otherwise obscure the relationships of interest to our study. We built our possible models by stepwise addition of each main effect and interaction. Each time a new term was added, we compared the new model to one without the additional term using likelihood ratio tests until we had identified the simplest model capturing significant

variance in the data. We used the normal approximation for determining parameter-specific p-values. In addition, we used Fisher's exact test to evaluate differences between treatments in the proportion of tadpoles surviving to metamorphosis.

Experiment 2: Relatedness and Size Asymmetry

To test our second set of predictions, we set up an experiment in which we paired two different-sized tadpoles, either from the same clutch or two different clutches. This allowed us to test whether potential chemical cues in the water could affect tadpole growth and development, differing based on perceived kinship and relative size asymmetry. Resources were held constant across treatments in this experiment.

We collected adult toad pairs in late April and early May of 2013 as described for the first experiment, resulting in a total of eight clutches from both the KBS Experimental Pond Laboratory and Lux Arbor Reserve.

To determine whether kinship and relative size interact to influence our fitness indices, we created containers to house two tadpoles in the same box while separating them by a screen. This allowed visual and waterborne cues to travel back and forth, giving tadpoles the opportunity to grow, develop, or change feeding habits in response to differences in cues if either individual had a fitness benefit to doing so, but prevented the larger tadpole from simply stealing food from the smaller tadpole. Boxes were set up in blocks of 60, for a total of 240 tadpoles, on May 28 and 29. Each tadpole's mass, SVL, and Gosner stage was measured before being added to one side of a container containing 600 mL of water. Tadpoles were paired with a sibling (kin treatment) or an individual from another clutch (non-kin treatment), with one tadpole designated as the

larger individual compared to its smaller box partner (Figure 2). All tadpoles received three pellets of food daily.

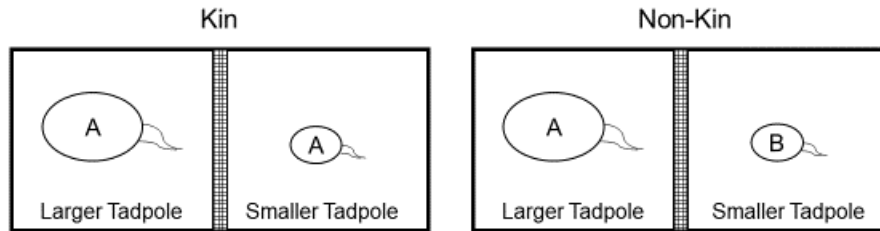


Figure 1.2. A schematic of the experimental design testing how focal tadpoles respond to cues of relatedness and size asymmetry. Two tadpoles, one larger in size than the other, were placed in the same container separated by a screen. In the kin treatment, both tadpoles were from the same clutch. In the non-kin treatment, each tadpole was from a different clutch.

We measured mass, SVL, and Gosner stage for each block of tadpoles every other day for approximately three weeks, to capture initial growth trajectories for each tadpole. After that point, tadpoles were checked daily and measured and removed if they had reached metamorphosis (Gosner stage 42). The first tadpole reached metamorphosis on June 22, while the final metamorph emerged on July 23. After 56 days, 221 tadpoles reached metamorphosis. 17 did not survive, and two survived the experiment but did not metamorphose.

Statistical Analysis

We tested whether kinship and relative size affected tadpole growth over time using the *lme4* package in R to fit the response variable of tadpole mass to the day that measurement was taken. We also looked at fixed effects of relative size (larger or smaller) and kinship (kin or non-kin), as well as interactions between these main effects. Random effects of individual tadpole ID and family were also included in the models to

account for variation in individual growth trajectories or differences among families that might otherwise obscure the relationships among the fixed effects. As in the previous experiment, we used stepwise addition of each main affect and interaction to build our possible models for consideration. We used likelihood ratio tests to compare each new model to one without the additional term until we determined the simplest model that captured significant variance in the data. We then used the normal approximation for determining parameter-specific p-values. In addition, we used Fisher's exact test to evaluate differences between treatments in the proportion of tadpoles surviving to metamorphosis.

Results

Experiment 1: Relatedness and Food Levels

Our best-fit model of tadpole growth rate included whether the tadpole survived to metamorphosis, family, kinship cue, and resource cue. We expected that whether a tadpole lived to metamorphosis may be an indicator of some underlying quality, which could then influence how quickly that tadpole grew over the course of the experiment. The model also included interactions between reaching metamorphosis and family, and family and kinship cue (Table 1.2). This was a significant improvement over the next simplest model ($\chi^2 = 7.88$, $p = 0.049$), indicating that the included parameters predict observed variation in tadpole development. All models included focal tadpole source tank and the water source tank as random intercepts.

Table 1.2. Models considered in the process of choosing the best-fit model by stepwise addition of each subsequent term. Each model's p-value represents the results of a likelihood ratio test comparing it to the next simplest model. The best-fit model was reached when no additional interactions resulted in a significant improvement.

Model	p-value
Growth Rate ~ Reached Metamorphosis + Family + Resource Cue + Kinship Cue + Family:Kinship Cue + Reached Metamorphosis:Family + (1 Tadpole Source Sank) + (1 Water Source Tank)	0.049
Growth Rate ~ Reached Metamorphosis + Family + Resource Cue + Kinship Cue + Family:Kinship Cue + (1 Tadpole Source Sank) + (1 Water Source Tank)	0.032
Growth Rate ~ Reached Metamorphosis + Family + Resource Cue + Kinship Cue + (1 Tadpole Source Sank) + (1 Water Source Tank)	0.89
Growth Rate ~ Reached Metamorphosis + Family + Resource Cue + (1 Tadpole Source Sank) + (1 Water Source Tank)	0.049
Growth Rate ~ Reached Metamorphosis + Family + (1 Tadpole Source Sank) + (1 Water Source Tank)	0.007
Growth Rate ~ Reached Metamorphosis + (1 Tadpole Source Sank) + (1 Water Source Tank)	1.16e-14
Growth Rate ~ 1 + (1 Tadpole Source Sank) + (1 Water Source Tank)	

We saw that the growth rates of isolated tadpoles with plentiful food were influenced by the cues of kinship (Fig. 1.3A) and resource abundance (Fig. 1.3B) they received in water. The patterns became clearer when examined by treatment combination. Tadpoles receiving cues of abundant resources had lower growth rates over the experiment than those that received cues of scarce resources (Fig. 1.3B). Consistent with our second hypothesis (Table 1.1), this difference was particularly pronounced when comparing tadpoles that did and did not survive to metamorphosis, especially for those receiving cues from kin (Fig. 1.3C, $t = -3.09$, $p = 0.002$). We also saw focal tadpoles that eventually reached metamorphosis had higher growth rates compared to those that did not (Fig. 1.3C, $t = 2.28$, $p = 0.02$). We did not see an effect of kinship cues on growth rates when looking at tadpoles that reached metamorphosis,

but for tadpoles that failed to metamorphose we found a kinship effect consistent with our second hypothesis (Table 1.1) that they grew more rapidly when receiving cues from kin with scarce resources than from non-kin with scarce resources (Fig. 1.3C, $t = -2.64$, $p = 0.008$).

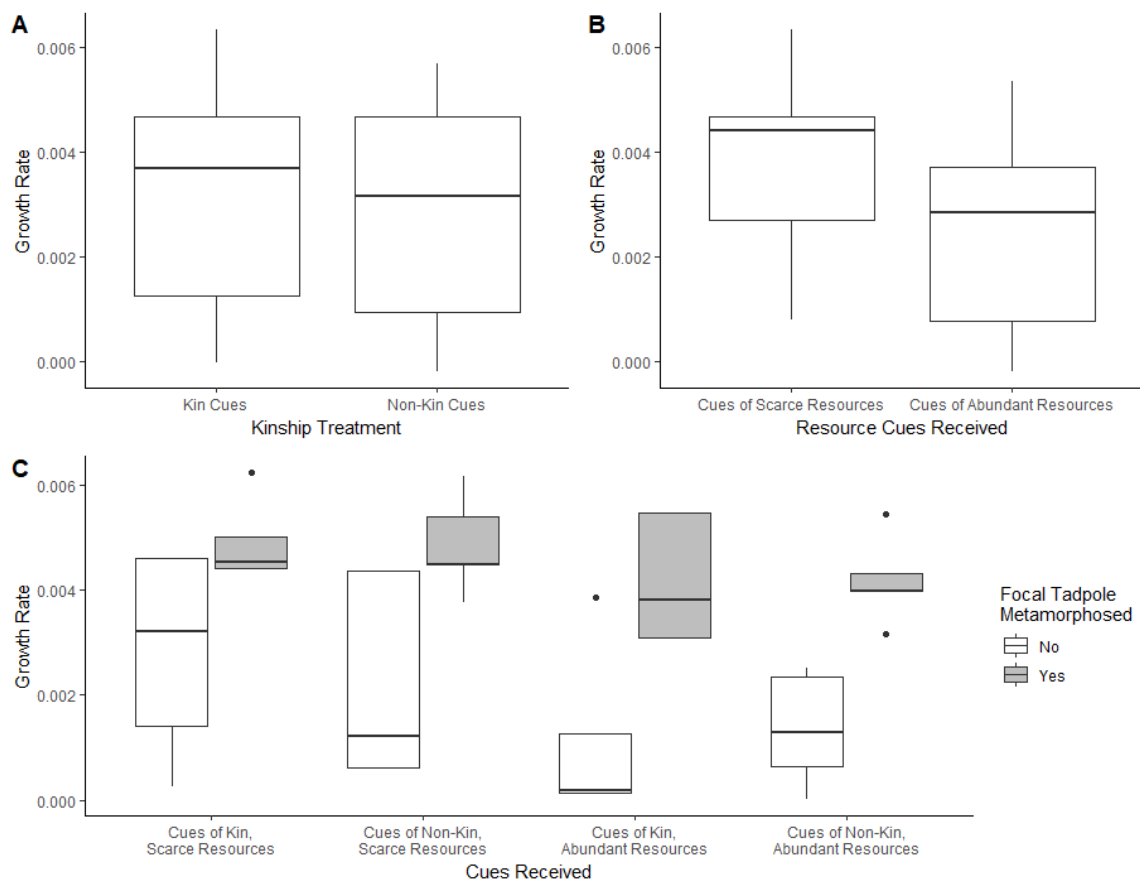


Figure 1.3. Relationship between focal tadpole growth rate and the cues in the water received by that tadpole. Panel A shows growth rates for all focal tadpoles receiving water from kin compared with tadpoles receiving water from non-kin. Panel B shows growth rate for all focal tadpoles receiving water from tanks with scarce resources compared with tadpoles receiving water from tanks with abundant resources. Panel C shows growth rates broken down by each combination of kinship and resource cues, as well as whether the focal tadpole eventually reached metamorphosis (boxes in gray indicate that they did metamorphose, while boxes in white indicate that they did not).

While growth rates did not differ significantly between kinship treatments for those that reached metamorphosis, we did see differences among treatments in the proportion of tadpoles that reached metamorphosis (Fig. 1.4). Overall, a greater

proportion of tadpoles survived to metamorphosis when receiving water from tanks with scarce than abundant resources ($p = 0.027$). Of the tadpoles in the abundant resource cue treatment, those receiving water from kin metamorphosed in similar proportions to those in the scarce resource cue treatment (Fig. 1.4A, $p = 0.36$), while those in the non-kin treatment were less likely to reach metamorphosis than those receiving cues of resource scarcity (Fig. 1.4B, $p = 0.044$).

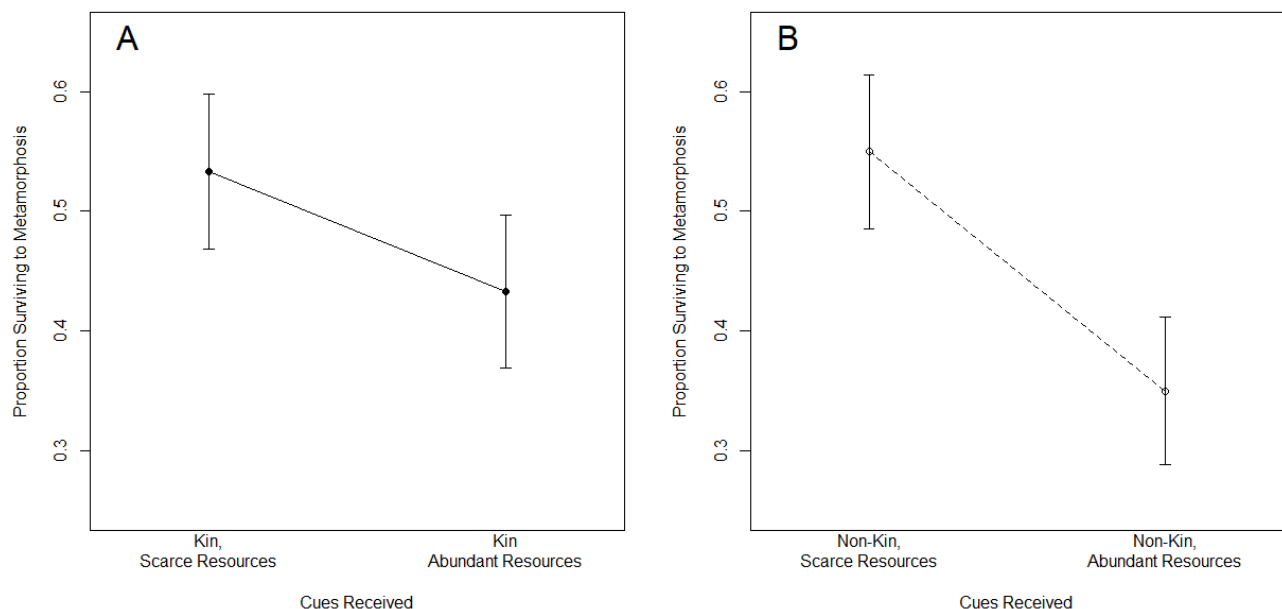


Figure 1.4. Proportion of focal tadpoles reaching metamorphosis based on resource cues (water from tanks with scarce or abundant resources) received for (A) tadpoles receiving cues from kin and (B) tadpoles receiving cues from non-kin. Error bars indicate the standard error of proportion.

Experiment 2: Relatedness and Size Asymmetry

Our best-fit model of tadpole mass contained time, relative size, family, and kinship as predictors, as well as interactions between size and family, size and time, time and family, family and kinship, and time, size, and kinship (Table 1.3). This was a

significant improvement over the previous model ($\chi^2 = 4.69$, $p = 0.09$), indicating that the included parameters predict observed variation in tadpole development. All considered models included random effects of focal tadpole identity on slope and intercept.

Tadpoles showed differences in growth in response to visual and chemical cues of relative size, with larger tadpoles outperforming smaller partners. At the beginning of the experiment, there was a significant size gap between tadpoles assigned to the “larger” and “smaller” treatments ($t = -10.44$, $p = 0.00$) but not between kinship treatments ($t = -0.22$, $p = 0.82$). We saw significant increases in mass over the initial period of the experiment for larger tadpoles (Fig. 1.5, $t = 25.76$, $p = 0.00$), with kinship treatment having no effect on growth patterns for these individuals (Fig. 1.5, $t = -0.08$, $p = 0.94$). Smaller tadpoles grew less rapidly than their larger partners overall (Fig. 1.5, $t = -2.72$, $p = 0.006$).

Contrary to what we saw in larger tadpoles, kinship treatment had a significant effect on smaller tadpoles, with individuals paired with non-kin growing even more slowly than those paired with kin (Fig. 1.5, $t = -2.14$, $p = 0.03$). Smaller tadpoles growing more rapidly if their partner was a sibling supports our second hypothesis (Table 1.1).

Over 90% of tadpoles in this experiment reached metamorphosis. Larger tadpoles were more likely than smaller tadpoles to reach metamorphosis ($p = 0.003$). Tadpoles paired with kin did not differ between size treatments in the proportion reaching metamorphosis ($p = 0.11$), while tadpoles paired with a non-sibling were more likely to reach metamorphosis if they were the larger of a pair ($p = 0.029$).

Table 1.3. Models considered in the process of choosing the best-fit model by stepwise addition of each subsequent term. Each model's p-value represents the results of a likelihood ratio test comparing it to the next simplest model. The best-fit model was reached when no additional interactions resulted in a significant improvement.

Model	p-value
Mass ~ Time + Relative Size + Family + Kinship + Relative Size:Family + Time:Relative Size + Time:Family + Family:Kinship + Time:Relative Size:Kinship + (1 + Time Tadpole ID)	0.09
Mass ~ Time + Relative Size + Family + Kinship + Relative Size:Family + Time:Relative Size + Time:Family + Family:Kinship + (1 + Time Tadpole ID)	0.04
Mass ~ Time + Relative Size + Family + Kinship + Relative Size:Family + Time:Relative Size + Time:Family + (1 + Time Tadpole ID)	0.003
Mass ~ Time + Relative Size + Family + Kinship + Relative Size:Family + Time:Relative Size + (1 + Time Tadpole ID)	9.08e-07
Mass ~ Time + Relative Size + Family + Kinship + Relative Size:Family + (1 + Time Tadpole ID)	1.37e-10
Mass ~ Time + Relative Size + Family + Kinship + (1 + Time Tadpole ID)	0.332
Mass ~ Time + Relative Size + Family + (1 + Time Tadpole ID)	<2.2e-16
Mass ~ Time + Relative Size + (1 + Time Tadpole ID)	<2.2e-16
Mass ~ Time + (1 + Time Tadpole ID)	<2.2e-16
Mass ~ 1 + (1 + Time Tadpole ID)	

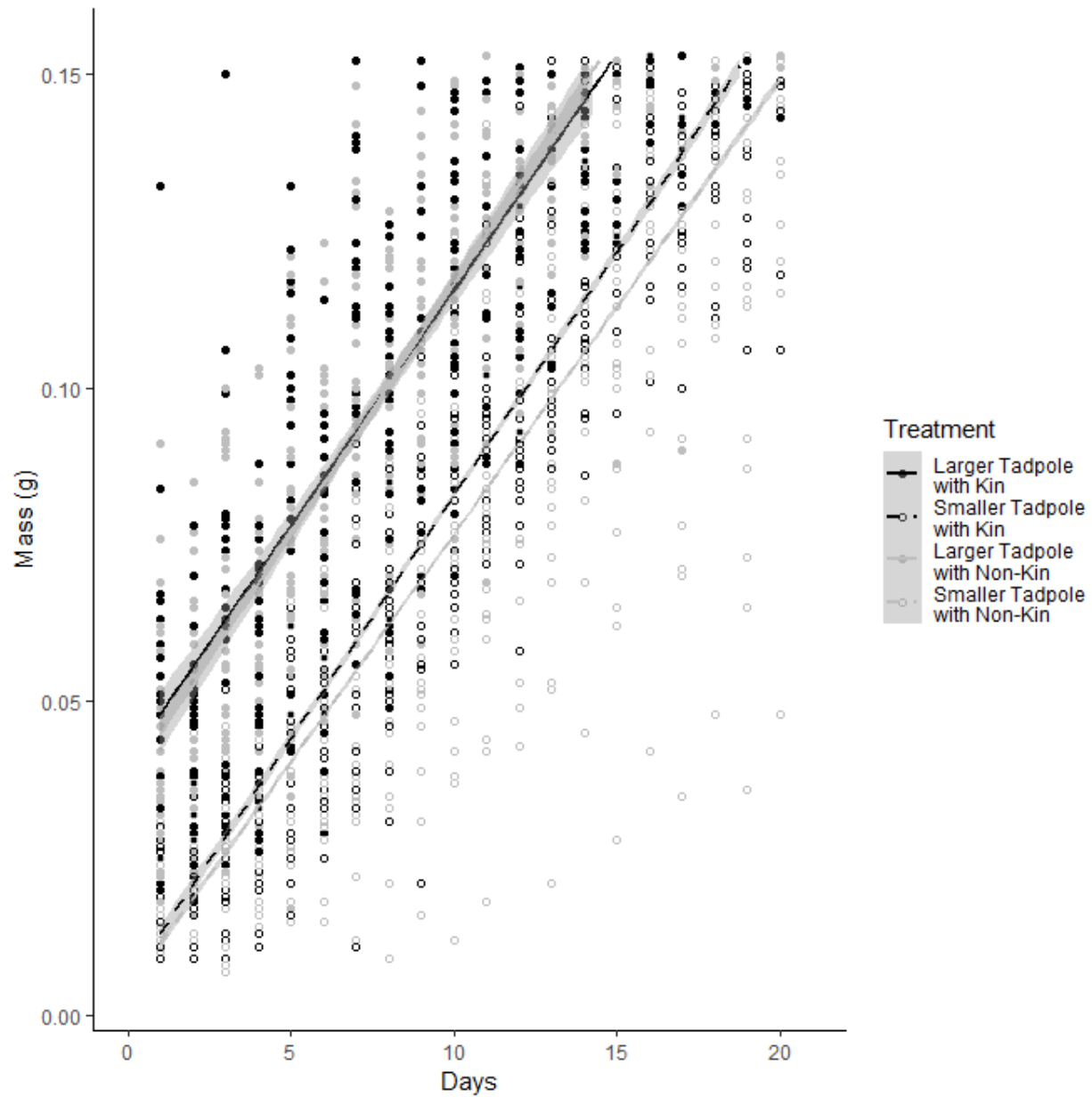


Figure 1.5. Relationship between time (in days) and mass (in grams) for tadpoles in a pair separated by a screen. Filled circles and solid lines indicate the larger tadpole of a pair (L), open circles and dashed lines indicate the smaller tadpole (S). Points in black indicate tadpoles paired with a sibling (K), points in gray indicate tadpoles paired with a non-sibling (N). Lines show the prediction from the full mixed-effects model ($\pm$ SE).

Discussion

We found evidence that tadpoles were able to perceive differences in the kinship and size of conspecifics based on water exposure alone, consistent with our predictions that chemical cues are sufficient to influence tadpole fitness. In our first experiment, we found that cues indicating conspecifics experiencing scarce resources resulted in more rapid growth of well-fed focal individuals than cues indicating abundant resources for conspecifics (agreeing with Table 1.1, H1, P1). We also found differences between kinship cue treatments in tadpoles who died before reaching metamorphosis (Table 1.1, H2, P1). In the second experiment, larger tadpoles grew more rapidly than paired smaller tadpoles. While kinship did not affect the growth rates of larger tadpoles, smaller tadpoles performed better when their partner was a sibling (Table 1.1, H2, P2). In both experiments, we saw variation in proportion surviving to metamorphosis, with tadpoles in the treatment resulting in slower growth being less likely to metamorphose when receiving cues from non-kin. This indicates that kinship can determine how the perceived environment influences fitness, consistent with our second hypothesis (Table 1.1).

It might seem counterintuitive that tadpoles in the first experiment grew more rapidly when receiving cues of scarcity, but the treatments refer to the resource levels in the tanks providing the focal tadpole's water. All focal tadpoles were fed at the abundant resource level, although they may use some type of cue in the water to inform them about resource availability to conspecifics. We hypothesize that tadpoles in the scarce resource cue treatment perceive their resource patch to be of higher quality than the surrounding environment. This perceived difference might lead them to harvest these

abundant local resources as quickly as possible, as personal survival in a “harsher” environment may trump any benefits of sharing those resources with kin (Pfennig et al. 1993).

Another possibility is that tadpoles in the abundant resource source tanks are producing more waste with more food and the resulting water is of poor quality. However, the differences between kinship treatments we saw for tadpoles that did not survive to metamorphosis may suggest they may not be simply failing to thrive. Tadpoles in most treatment combinations showed similar patterns of growth over time regardless of whether they reached metamorphosis, with a tendency for slower-growing tadpoles to fail to metamorphose. Those receiving water from kin in the abundant resource treatment, on the other hand, did not show an increase in mass over time over the course of the experiment, despite starting out at similar sizes as tadpoles who failed to metamorphose in other treatments (Fig. 1.3C). This is consistent with the hypothesis that tadpoles who are smaller and unlikely to reach metamorphosis may receive a greater inclusive fitness benefit from leaving resources for kin instead of competing for resources and attempting to reach metamorphosis themselves.

Although it may seem costly to be around kin in these circumstances, we found a fitness benefit. While an overall greater proportion of tadpoles reached metamorphosis when receiving water from tanks with scarce than abundant resources (54% vs. 39%), we saw that there was no significant difference in survival to metamorphosis between resource cue conditions when looking only at tadpoles in the kin cue treatments (Fig. 1.4). In natural populations, the proportion of tadpoles that reach metamorphosis when around kin may be further increased by siblings who were unlikely to reach

metamorphosis themselves competing less intensely for available resources. This is consistent with previous work showing that there is an inclusive fitness benefit to being around kin even for those individuals who are unlikely to successfully compete (Pfennig 1997, Dugas et al. 2016).

In the second experiment, we saw that larger tadpoles in a pair did not appear to be affected by kinship treatment, but smaller tadpoles grew more rapidly when their partner was a sibling (Fig. 1.5). A chemical mechanism seems likely; as tadpoles were separated by screens, this cannot simply be due to larger tadpoles competing more intensely with unrelated individuals for available food. This suggests that previously seen patterns of larger tadpoles suppressing the growth of smaller tadpoles (Light 1967, Steinwascher 1978, Griffiths et al. 1993) may be sensitive to specific environmental factors rather than being a uniform phenomenon.

If larger tadpoles do not uniformly suppress the growth of smaller tadpoles, this raises questions about how this can be avoided between kin. It is possible that tadpoles may be able to avoid releasing the chemical cues responsible for growth suppression when surrounded by kin, but another alternative is that smaller tadpoles are able to avoid responding to sibling cues. Other toad species produce a greater variety of bufotoxins, not just greater amounts, as the density of conspecifics increases (Bókonyi et al. 2016). If variation in bufotoxin composition has a genetic component, kin who are more genetically similar may also produce more similar toxins, which may have an immune function (Bókonyi et al. 2018). Tadpoles are unlikely to respond to compounds similar to “self,” suggesting a mechanism for avoiding suppression by larger kin.

In addition to these changes in early growth patterns, we also saw some variation in the proportion of tadpoles reaching metamorphosis across treatments. For tadpoles paired with a non-sibling, they were more likely to reach metamorphosis if they were the larger rather than the smaller of the pair. In both experiments, tadpoles showed greater differences between treatments in survival to metamorphosis when the cues they received came from non-kin. The fact that this pattern exists given only exposure to water and not direct interaction between individuals indicates that waterborne chemical cues are an important component of fitness benefits from kin aggregations.

Tadpole growth and development varies with differences in environmental conditions; these experiments indicate that their perceptions of their environment are influenced by chemical cues in the water. Physical interactions with conspecifics and the surrounding habitat are necessary for understanding the full picture, but we have seen that exposure simply to water from different conditions communicates information about relatedness and relative size of conspecifics as well as resource availability.

These differences influence the proportion of tadpoles surviving to metamorphosis as well as altering how quickly they get there, both of which affect fitness. Responding to variation in the environment requires tadpoles to be able to perceive this variation; understanding the role of chemical cues allows us to make sense of how variation in more complex environmental scenarios results in changes in fitness.

CHAPTER TWO:
***THE BENEFITS OF DEVELOPING WITH KIN DEPEND ON DENSITY AND
NUTRIENT AVAILABILITY IN THE AMERICAN TOAD (ANAXYRUS AMERICANUS)***

Introduction

Siblings can be an organism's most intense competitors or its most reliable allies, depending on the circumstances. The development of kin selection theory and the concept of inclusive fitness provided a theoretical framework to help us understand the shifting interplay of competition and cooperation among individuals with more or less genetic similarity (Gardner and West 2014). Hamilton's rule (Hamilton 1964) provides a framework for us to think about how the evolutionary optimum between selfishness and cooperation among related individuals might be reached. Since its formulation, most studies have focused on how the relatedness of interacting individuals impacts the evolution of cooperation, without much consideration to variation in the contexts in which these interactions occur. More recently, researchers have begun to explore how context-dependencies can alter the costs and benefits of cooperation, which appears to be an important part of the story (Bourke 2014).

Parker et al. (1989) showed how changes in the relative marginal benefits of a resource can change the level of conflict between siblings. Differences in size among siblings can be an important factor in these competitive relationships. When the benefits of increasing condition or size show diminishing returns (a concave fitness function, as in Jensen's inequality in economics, Fig. 2.1A) and the marginal fitness benefits of the resource to the larger (or better condition) full sibs are less than twice the marginal benefits to the smaller (poor condition) sib, the larger full sib will increase its inclusive fitness more if the smaller individual is able to increase in size (Fig. 2.1B)

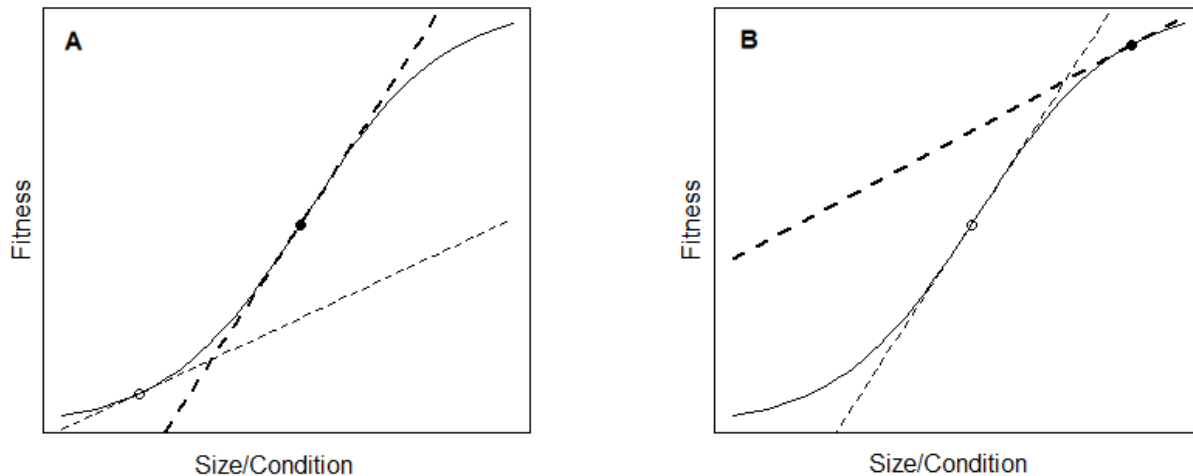


Figure 2.1. A sigmoidal relationship between size or condition and fitness, where growth results from acquisition of limited resources. Dashed lines indicate the marginal fitness benefit that individuals receive from an increase in size or condition given their current state. Filled circles and thicker dashed lines indicate the larger individual while open circles with thinner dashed lines indicate the smaller individual. If the marginal benefit of a resource to a larger sibling is more than twice that of a smaller sibling, the smaller sibling should yield that resource (A). When the marginal benefit to the smaller sibling is more than twice that of the larger sibling, the larger sibling should yield instead (B).

Extending that logic, accelerating benefits of resource acquisition in very poor condition or size create a convex fitness function. This creates the possibility that the marginal fitness benefit of a resource to the larger sib is more than twice the benefit to the smaller sib. Given an average relatedness of 0.5 between full sibs, according to Hamilton's rule, the smaller sib should then yield resources to the larger (Fig. 2.1A). This idea seems to be the foundation for the recent theoretical analysis by Thompson et al. (2017), showing that dominant sibs are sometimes selected to exploit weaker sibs because they are less resistant to exploitation than are unrelated individuals. If the fitness function is concave at low size or condition and convex at high size or condition, the resulting sigmoid fitness function (see Smallwood 1996 Fig. 5 and references) could select for the small sib to defer to the larger sib early on and then the larger sib might

defer to the smaller later. Patterns that look like “deferral” may be the result of dominant individuals selectively exploiting their weaker relatives or individuals competing less intensely for (or yielding) resources to a relative. Both could result in increased inclusive fitness for all individuals and should be common among sibs of different size.

Unfortunately, measuring the marginal fitness benefit of a resource to siblings of differing size is challenging, particularly for seasonally-breeding vertebrates like the ones that inspired Parker’s model, and where any given unit of the resource results in a relatively small increase in fitness. Consequently, many well-known examples of kin-directed altruism center on cases where the costs are clear-cut and dramatic, as in cases of cannibalism or when an individual puts itself at risk to warn relatives (Sherman 1977, Pfennig et al. 1993, Joseph et al. 1999, Pfennig 1999, Beavis et al. 2007). There are, however, many conditions in which individuals must account for more subtle costs of cooperation when interacting with kin, especially given limited methods for minimizing direct competition (Lambin et al. 2001). These scenarios require different strategies to evolve to maximize inclusive fitness. Theory predicts that individuals might adjust how intensely they compete for resources according to how related they are; for example, smaller siblings will increase inclusive fitness by deferring to larger, healthier kin, while in other cases larger siblings increase inclusive fitness by allowing smaller kin primary access to food (Parker et al. 1989, Yamamura and Higashi 1992). Studies from a variety of taxa do in fact show sibling interactions conforming to these predictions (Willson et al. 1987, Tonsor 1989, Briskie et al. 1994, Johnstone and Roulin 2003, Boncoraglio et al. 2009, Madden et al. 2009, File et al. 2011). We predict similar conditions should occur in amphibians, which would offer novel opportunities to

determine how environmental heterogeneity can induce and modulate kin effects on development.

Because larval amphibians cannot leave their ponds until metamorphosis, they are likely to interact with other individuals present in the same water body. Depending on population densities and distributions, local conspecifics might originate from one clutch of eggs, or from clutches of many different females. Some species of larval amphibians form aggregations, often composed of siblings (Waldman and Adler 1979, Waldman 1982, Waldman 1986, Pfennig et al. 1993), which may further structure the relatedness of individuals that interact during development. These aggregations provide many benefits, such as anti-predator defense and social foraging (Watt et al. 1997, Sontag et al. 2006), but their costs are more complicated, particularly under varying levels of local resource competition.

A variety of population responses have been seen when tadpoles interact at high densities (Steinwascher 1979, Breden and Kelly 1982, Semlitsch and Caldwell 1982, Travis and Trexler 1986, Warner et al. 1991, Newman 1994), but one common trend is for larger tadpoles to inhibit the growth of smaller conspecifics when competing for limited resources, presumably through use of a waterborne chemical signal (Light 1967, Steinwascher 1978, Griffiths et al. 1993). Relatedness has sometimes been considered in experiments looking at growth and development (Hokit and Blaustein 1994, Hokit and Blaustein 1997, Saidapur and Girish 2001, Pakkasmaa and Aikio 2003, Gramapurohit et al. 2008), but mixed results have been found, and it is not always clear how kin recognition mechanisms might interact with density and growth suppression.

The American toad (*Anaxyrus americanus*) is one species that shows evidence of aggregating with kin in its larval stage, which is likely facilitated by an olfactory mechanism (Waldman and Adler 1979, Waldman 1982, Waldman 1986). Unlike some other species that recognize kin, there is no evidence that American toads actively cannibalize one another (Heinen and Abdella 2005), suggesting that they must receive other benefits from being able to distinguish kin from non-kin. *A. americanus* are explosive breeders, with mating and egg-laying taking place within a relatively small window in the spring, who use water bodies ranging from small temporary pools to large permanent ponds. This means that there can be a great deal of natural variation in tadpole density, and potentially relatedness, within a pond. American toads are therefore well suited for testing questions of how relatedness influences fitness and whether this relationship is context-dependent.

We investigated whether relatedness would influence growth and development in experimental groups of tadpoles, and whether other factors – such as density and nutrient availability – would impact the fitness benefits of grouping with kin. We predicted that tadpoles in less competitive environments (e.g. lower density, higher nutrient availability) would metamorphose at their theoretically optimal size (Collins 1979), regardless of how long they develop, as they are not constrained by competition for resources. In contrast, we expected tadpoles reared in competitive, resource-limited conditions would exhibit a trade-off between rate and size at metamorphosis, since early-emerging individuals will leave comparatively more competitive environments than late developing counterparts, assuming resource productivity is constant. If tadpoles do not suppress the growth of siblings or spend less time engaged in interference

competition with them, we expect that tadpoles in a pure kin environment will outperform individuals in mixed groups. Such a benefit from grouping with kin may even counteract the costs that come from potentially increased competition when density is higher or resources are more limited. It is also possible that more competitive environments will cause the costs of deferring to kin to outweigh the benefits, causing kin instead to behave more selfishly in order to maximize direct fitness.

As a result, we predicted that (1) there will be a difference in size at metamorphosis between kin and mixed treatments, (2) tadpoles will be larger at metamorphosis in low density and higher nutrient availability treatments, and (3) the effect of kinship will vary depending on density and resource availability. By manipulating relatedness, resource availability, and population density in one experiment, we hoped to determine not just whether these factors matter individually, but how the interactions between them affect fitness.

Methods

To test these predictions, we set up a cattle tank experiment in which we manipulated the relatedness and density of tadpole groups and the nutrient availability in the tank. Adult American toads were collected during breeding choruses in late April of 2016. We collected pairs in amplexus from several locations at the Kellogg Biological Station (KBS) Experimental Pond Laboratory and Lux Arbor Reserve in southwest Michigan. Each pair was isolated in a bucket for approximately 24 to 48 hours until they finished laying their eggs, at which point they were returned to the location at which they were captured. We then transferred each clutch to a 20-gallon aquarium tank, until the

tadpoles reached a Gosner developmental stage of at least 26 (Gosner 1960), after which they are free-swimming and able to feed independently.

We then set up 18 cattle tanks outdoors at the Pond Laboratory. Each tank contained approximately 75 L of water, with each tank being randomly assigned to treatments (pure or mixed kin, high or low tadpole density, and with or without nutrient enrichment). Each tank was seeded with pond water to promote the growth of periphyton to serve as a food source for tadpoles. In addition to this basic tank setup, half of the tanks received augmented nutrient levels ($\text{NaNO}_3\text{-N}$ 160 $\mu\text{g L}^{-1}$, KH_2PO_4 10 $\mu\text{g L}^{-1}$) as described in Wood and Richardson 2009. We added an additional nutrient pulse every two weeks in order to create and maintain a nutrient-enriched environment with more plentiful resources. Tanks were populated with either pure kin – containing tadpoles from only one sibship – or mixed kin – containing tadpoles from two sibships. High density tanks contained 100 tadpoles, while low density tanks contained 50 tadpoles. These densities are within the range seen in natural tadpole populations based on personal observation. A total of 1350 tadpoles were introduced to the cattle tanks.

Before starting the experiment, we recorded each tadpole's mass, snout-vent length (SVL) and Gosner stage and placed it in the appropriate tank. All tadpoles in a given tank were introduced on a single day between May 31 and June 4. The date on which tadpoles were added was considered the start date for that particular tank, and larval period was measured from that date. We monitored each tank daily, removing tadpoles once they had reached metamorphosis, defined as Gosner stage 42 (at least one forelimb visible). Metamorphosed tadpoles had their mass, SVL, and Gosner stage

recorded, as well as the number of days that had passed since they were added to the tank. Size at metamorphosis is a common fitness proxy in studies of larval amphibians (Earl and Whiteman 2015), and larger size also increases toad survival in the terrestrial environment (Sams and Boone 2010). After 105 days, 1148 tadpoles reached metamorphosis. 196 did not survive, and six survived the experiment but did not metamorphose; these individuals were excluded from assessments of mass at metamorphosis and time to metamorphosis.

Statistical Analysis

We used the *lme4* package (Bates et al. 2015) in R (R Core Team 2017) to test the effects of kinship and other environmental factors on the mass of emerging tadpoles. Specifically, we fitted our response variable (mass at metamorphosis) to fixed effects of time to metamorphosis, density, nutrient addition treatment, and kinship, as well as interactions between these main effects. We also included family effects as random intercepts in the models, to account for any differences among clutches that might otherwise obscure the relationships of interest to our study. We built our possible models by stepwise addition of each main effect and interaction. Each time a new term was added, we compared the new model to one without the additional term using likelihood ratio tests until we had identified the best model capturing significant variance in the data. We considered this model to be reached when no additional terms resulted in a significant improvement to the model. We used the normal approximation for determining parameter-specific p-values.

Results

Predictors of larval development

Our best-fit model of mass at metamorphosis contained time to metamorphosis, density, nutrient addition, and kinship, as well as two-way interactions between time to metamorphosis and kinship, density and nutrient treatment, and a three-way interaction between time to metamorphosis, kinship, and nutrient treatment (Table 2.1). This was a significant improvement over the next simplest model ($\chi^2 = 9.79$, $p = 0.007$), indicating that all the included parameters are predictive of observed variation in tadpole development. In all models, family was included as a random effect.

Effects of kinship on development

In pure kin treatments, tadpoles that took longer to reach metamorphosis were also larger at metamorphosis than those with shorter larval periods (Fig. 2.2, $t = 9.63$, $p = 0.00$). We saw the opposite relationship in tanks containing mixed kin groups (Fig. 2.2, $t = -5.19$, $p = 2.03e-7$). The kinship treatment on its own did not predict size at metamorphosis independently of these interactions with larval period ($t = 0.75$, $p = 0.45$), and/or density and nutrient availability (see below).

Effects of density on development

Tadpoles in the low density treatment were larger at metamorphosis than tadpoles raised at high density (Fig. 2.3, $t = 2.98$, $p = 2.8e-3$), but we did not find that adding an interaction with larval period and density treatment significantly improved our model of size at metamorphosis ($\chi^2 = 1.25$, $p = 0.26$).

Effect of nutrient availability on development

The relationship between larval period and size at metamorphosis was significantly weaker in nutrient enriched treatments (Fig. 2.4, $t = -2.87$, $p = 4.1e-3$), resulting in late-metamorphosing tadpoles reaching similar sizes at metamorphosis regardless of the nutrient treatment.

Combined effects of kinship, density, and resource availability on development

We also found that the effect of kinship varied depending on the context. In addition to the overall relationship between time to metamorphosis and kinship treatment, we found an additional interaction between these variables and the nutrient treatment. At high densities with no nutrient addition we saw the greatest difference between pure kin and mixed kin treatments (Fig. 2.5A). Tadpoles grouped with siblings accumulated mass faster, and also reached metamorphosis earlier, than counterparts in mixed kin tanks. Tadpoles also see a benefit in increased size at metamorphosis in the low density tanks with no nutrient addition treatment (Fig. 2.5C). While significant differences between pure and mixed kinship treatments were seen with no nutrient addition (Fig. 2.5A, Fig. 2.5C), we found no significant difference between kinship treatments when more resources were available (Fig. 2.5B, Fig. 2.5D, $t = 0.44$, $p = 0.66$). We did not find any significant interactions between density and these other factors.

Table 2.1. Models considered in the process of choosing the best-fit model by stepwise addition of each subsequent term. Each model's p-value represents the results of a likelihood ratio test comparing it to the next simplest model. The best-fit model was reached when no additional interactions resulted in a significant improvement.

Model	p-value
Mass ~ Time to Metamorphosis + Density + Nutrient + Kinship + Kinship:Time to Metamorphosis + Density:Nutrient + Kinship:Time to Metamorphosis:Nutrient + (1 Family)	0.007
Mass ~ Time to Metamorphosis + Density + Nutrient + Kinship + Kinship:Time to Metamorphosis + Density:Nutrient + (1 Family)	0.07
Mass ~ Time to Metamorphosis + Density + Nutrient + Kinship + Kinship:Time to Metamorphosis + (1 Family)	3.24e- 05
Mass ~ Time to Metamorphosis + Density + Nutrient + Kinship + (1 Family)	0.09
Mass ~ Time to Metamorphosis + Density + Nutrient + (1 Family)	0.04
Mass ~ Time to Metamorphosis + Density + (1 Family)	1.51e- 15
Mass ~ Time to Metamorphosis + (1 Family)	<2.2e- 16
Mass ~ 1 + (1 Family)	

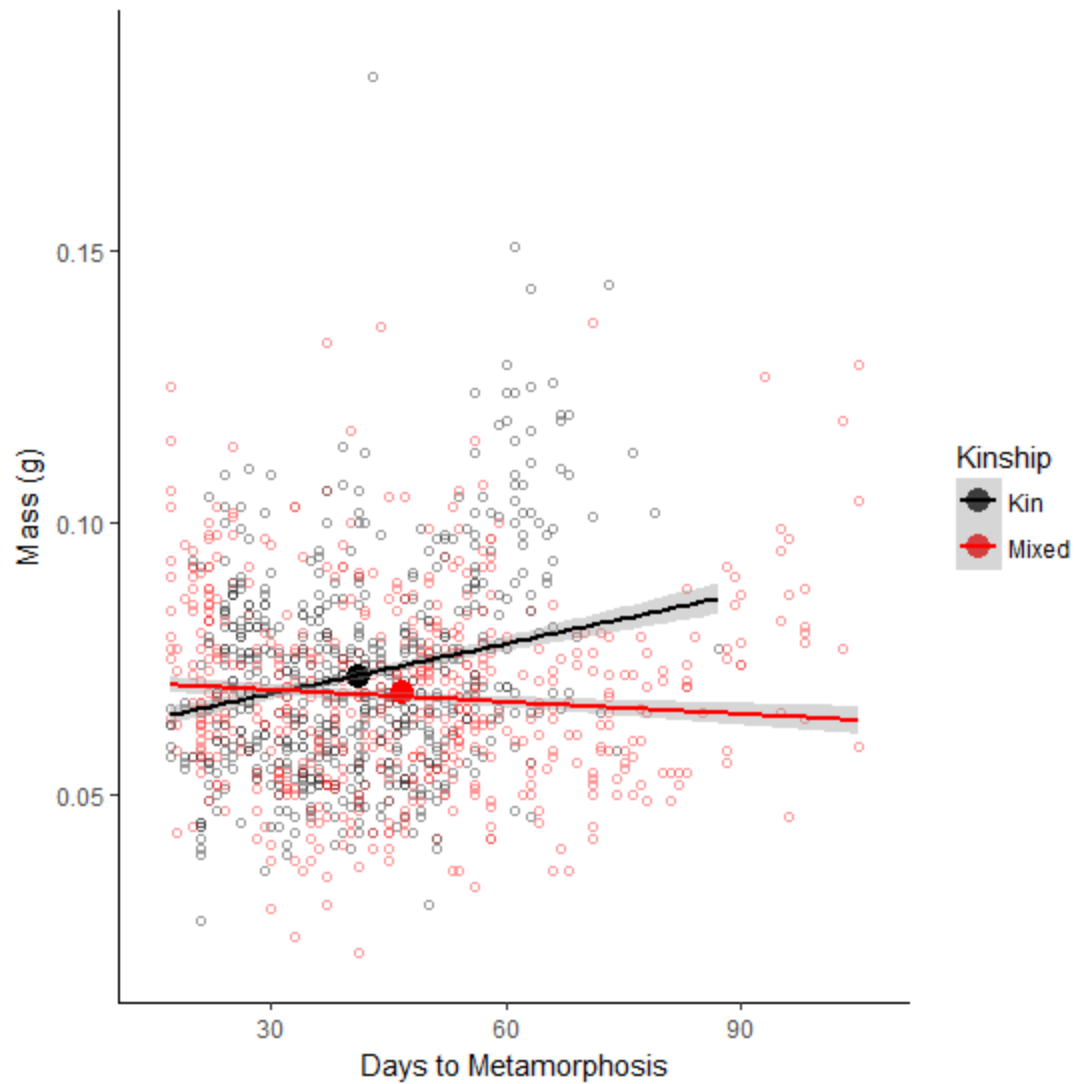


Figure 2.2. Relationship between time to metamorphosis (in days) and mass at metamorphosis (in grams) for the pure kin and mixed kin treatments. Lines show the prediction from the full mixed-effects model ($\pm$ SE). Larger points indicate the treatment means.

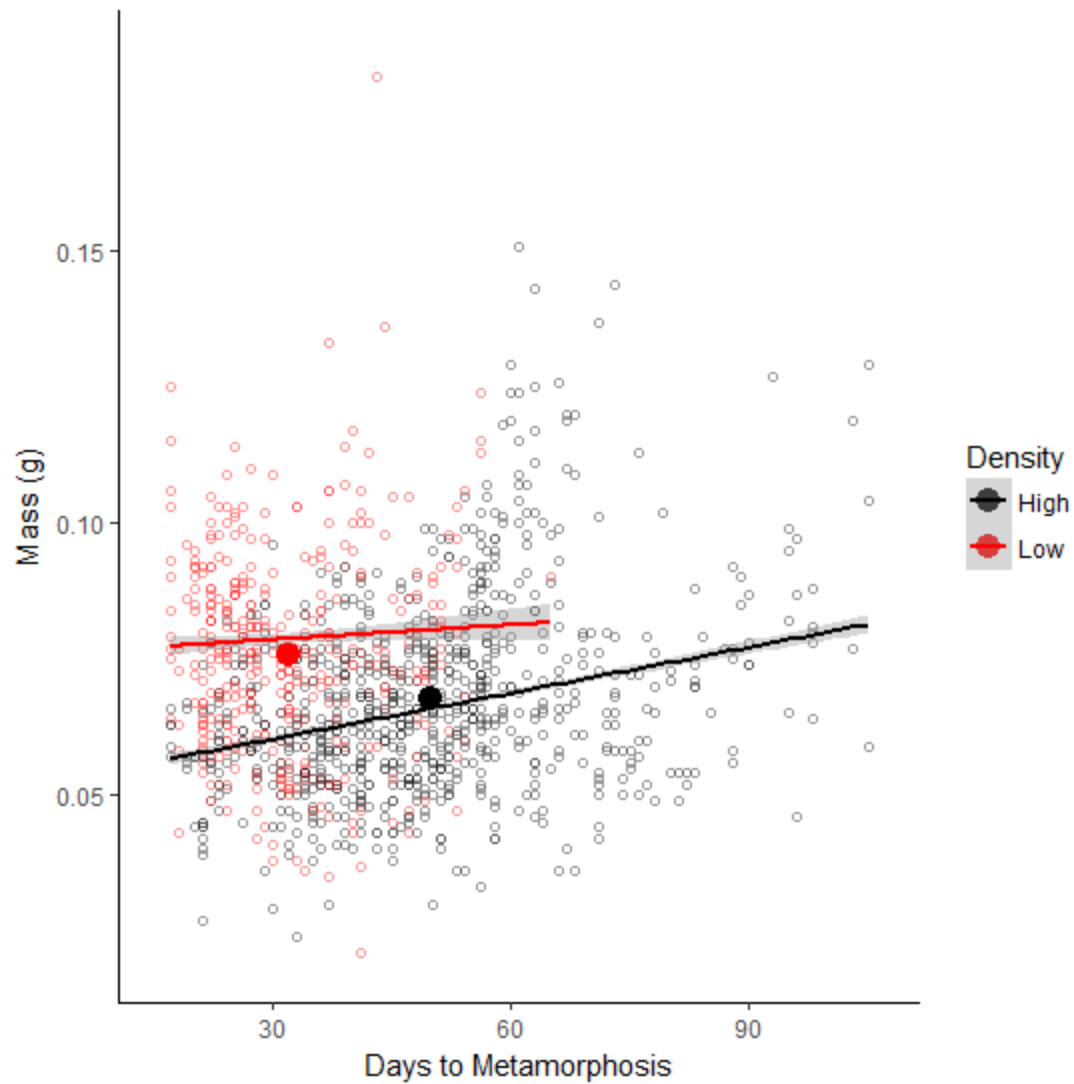


Figure 2.3. Relationship between time to metamorphosis (in days) and mass at metamorphosis (in grams) for the low density and high density treatments. Lines show the prediction from the full mixed-effects model ($\pm$ SE). Larger points indicate the treatment means.

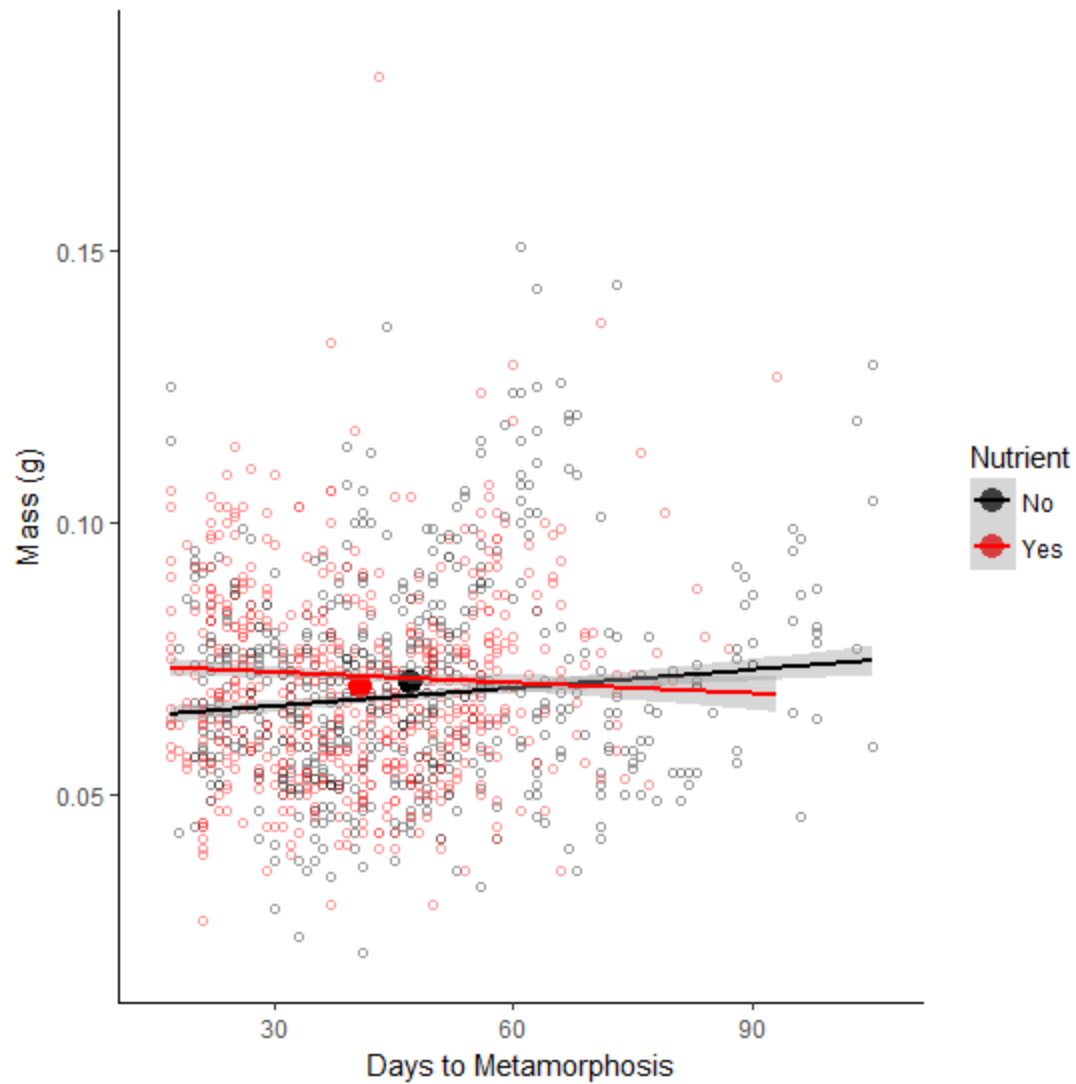


Figure 2.4. Relationship between time to metamorphosis (in days) and mass at metamorphosis (in grams) for the treatments with and without nutrient enrichment. Lines show the prediction from the full mixed-effects model ($\pm$ SE). Larger points indicate the treatment means.

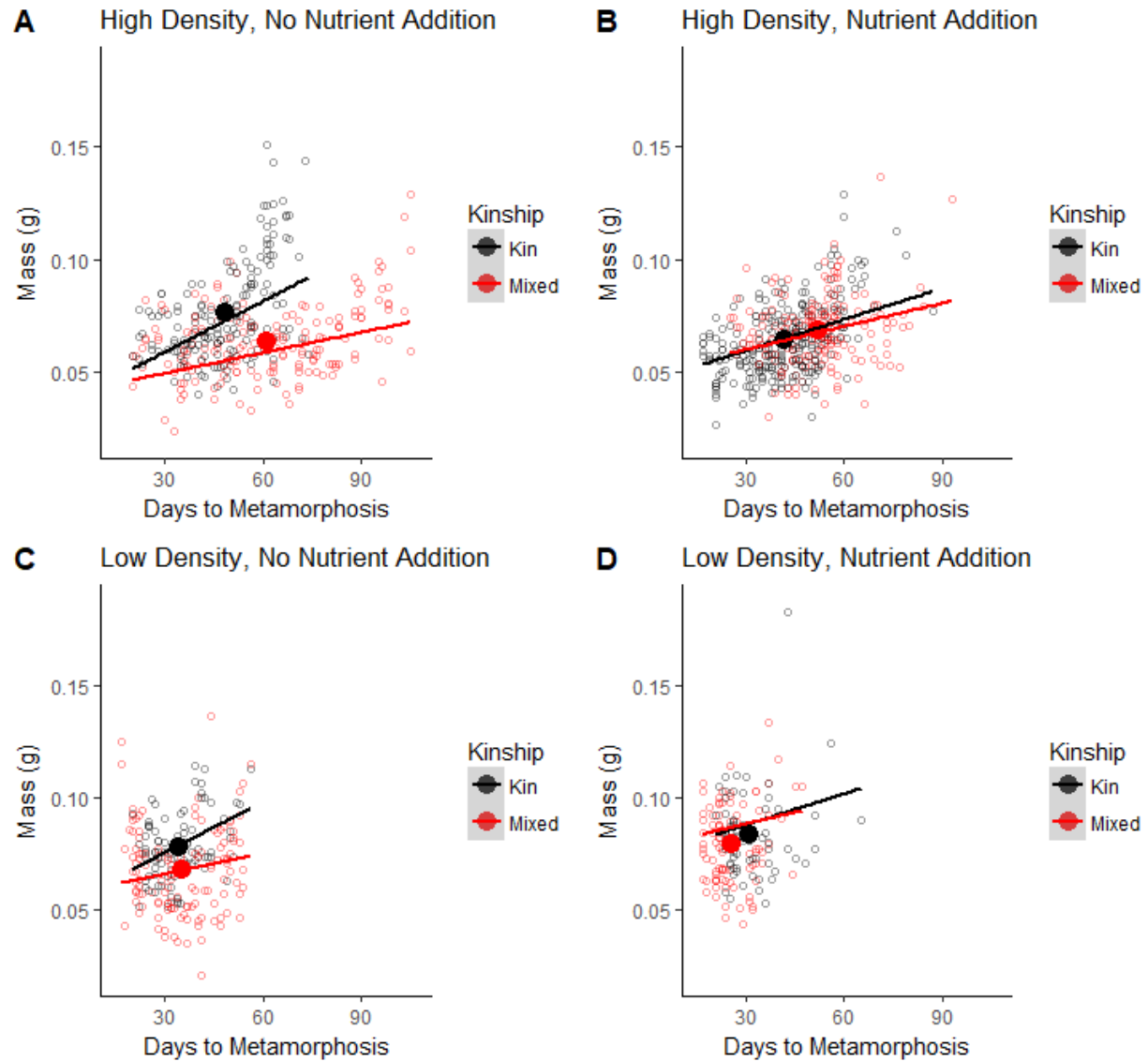


Figure 2.5. Relationship between time to metamorphosis (in days) and mass at metamorphosis (in grams) for the pure kin and mixed kin treatments for (A) high density with no nutrient addition, (B) high density with nutrient addition, (C) low density with no nutrient addition, and (D) low density with nutrient addition. Lines show the prediction from the full mixed-effects model. Larger points indicate the treatment means.

Discussion

We found strong evidence that 1) kinship affects aspects of development that are likely important for the fitness of *A. americanus* tadpoles and 2) the nature of these effects is highly context-dependent. First, we found an overall trend for tadpoles to reach larger sizes over a longer larval period in the pure kin treatment, while time did not predict size at metamorphosis in mixed kin treatments (Fig. 2.2). The fact that the duration of the larval period is not a strong predictor of size at metamorphosis in mixed kin treatments is consistent with Collins's (1979) predictions if interference competition is playing a larger role there than in pure kin treatments. Collins (1979) suggests that this pattern can emerge because at any given time, increased interference competition results in larger, more competitive tadpoles metamorphosing as smaller, less competitive tadpoles are metamorphosing. As a result, we do not see any overall trend in size at metamorphosis over time. This could result from chemical suppression, where larger tadpoles suppress the growth of smaller individuals, or physical interference, where individuals are directly interfering with access to resources. While further study is needed to distinguish between these (non-exclusive) explanations, our results suggest that, overall, tadpoles may be experiencing less interference competition from siblings than from unrelated individuals.

Additionally, we found that tadpoles were larger at metamorphosis in the low density treatments and that individuals metamorphosing earlier were larger in the nutrient addition treatment, but this difference disappeared with longer larval periods. Results from the nutrient-enriched treatments were consistent with the predictions of Collins (1979). Tadpoles whose tanks did not receive additional nutrient enrichment

were larger at metamorphosis when they took longer to develop, while larval period did not predict size at metamorphosis in tanks with higher nutrient availability (Fig. 2.4).

When resources are limited, we expect that the metamorphosis and departure of rapidly developing individuals will free up resources that allow remaining tadpoles to grow to larger sizes. In contrast, if resource levels are consistently high, and do not limit growth or development, there should be little difference in the size at metamorphosis of early vs. late-developing tadpoles. Overall, tadpoles raised at low density reached metamorphosis at larger body sizes than those raised at high density, but with similar increases in mass over time (Fig. 2.3).

While these overall trends are visible, we also found that environmental context influences the degree to which kinship drives differences in size at metamorphosis, which is a common proxy for fitness in developing tadpoles. We saw the greatest differences between kinship treatments in conditions where competition is likely more intense, given that more tadpoles are making use of less abundant resources (Fig. 2.5A, Fig. 2.5C). The comparative benefits of kinship disappear, however, in the nutrient enriched treatments (Fig. 2.5B, Fig. 2.5D), particularly at low density. When resource competition is less intense, refraining from interference competition to benefit kin does not provide enough of an additional fitness benefit to elicit cooperative strategies. Instead, strategies that maximize direct fitness are favored.

Previous work has found that larger tadpoles might suppress the growth of smaller tadpoles using chemical inhibition, a form of interference competition that can operate even without physical contact between individuals (Light 1967, Steinwascher 1978, Griffiths et al. 1993, Crossland and Shine 2012, Bókony et al. 2016). Based on

our results, chemical inhibition may be occurring in mixed sibship groups, where larger tadpoles do not receive a fitness benefit from deferral to smaller ones. This may explain why we see that time to metamorphosis is typically a poor predictor of size at metamorphosis in mixed kin groups. In pure kin treatments, by contrast, larger tadpoles may avoid suppressing the growth of smaller kin. This would mirror previous findings from other species, in which smaller individuals also benefit more from being grouped with kin (Jasienski 1988, Smith 1990).

Much work looking at kin discrimination in larval amphibians has been directed at species where there is strong potential for cannibalism. The advantage of such systems is that the costs and benefits considered under Hamilton's rule are clear. One tadpole receives a meal, while the cannibalized tadpole loses its life, bringing its direct fitness to zero. In these systems, it is clear that selection can promote kin discrimination, thereby minimizing the inclusive fitness costs of cannibalizing kin, while taking advantage of the benefits of cannibalizing non-related individuals (Pfennig 1997). In contrast to this scenario, *A. americanus* seems to better represent a case where the cost-benefit ratio is not so straightforward, which is likely most common in nature. It is therefore important to understand how and whether kin selection operates in these situations, and whether the resulting outcomes are a matter of degree, with kin discrimination acting more weakly but in the same manner, or kind, with less drastic costs leading to kin discriminating behaviors acting in a different way. It is also likely that the costs-benefits ratio will be more dependent on environmental context when direct fitness costs are not as drastic, leading to the variation we see across treatments.

These more subtle cases are also important to consider because frequent interactions with kin or the ability to discriminate kin from non-kin do not automatically result in cooperation. If kin tend to be more similar to one another in the types of resources they use or their ability to take advantage of and hold those resources, we may see more intense competition between relatives than non-relatives, particularly if diverse resources are not available for niche partitioning (Martin and Garnett 2013, Foster and Briffa 2014). Related individuals may also be targeted for aggression if they are less likely to resist kin (Thompson et al. 2017), and individuals with low fitness potential may even benefit from an inclusive fitness perspective by being cannibalized (Pfennig 1997, Dugas et al. 2016). The present study confirms the importance of considering how environmental factors influence the costs and benefits of competing or cooperating with kin and how this is likely to shape the resulting behaviors.

Our findings suggest that complex interactions between population structure and local environments are likely to shape the evolution of both kin discrimination, and the ways it is used to maximize inclusive fitness. The effects likely operate not only in American toads, but also in other species that can interact with both relatives and non-relatives during development. We suggest that further studies that isolate, and manipulate, both relatedness and ecological contexts will offer powerful insights into the evolution of kin recognition, cooperation, and their modulation of inclusive fitness.

CHAPTER THREE:
***DOES THE PRESENCE OF PREDATOR CUES ALTER AGGREGATION
PREFERENCES IN AMERICAN TOAD (ANAXYRUS AMERICANUS) TADPOLES?***

Introduction

Predation acts as a strong pressure on many aspects of animal biology, including selecting for warning coloration (Stevens and Ruxton 2012) or camouflage (Merilaita et al. 2017), inducing and selecting for changes in body shape or size (Price et al. 2015), altering foraging behavior and activity level (Lima and Dill 1990), and influencing other aspects of phenotype and behavior (Caro 2005). One aspect that can be strongly shaped by predation pressure is grouping behavior. Animals may come together into groups of varying size to benefit from increased vigilance and alarm calls or increased ability to defend against potential predators (Edmunds 1974). In addition to these strategies, there are other ways that joining larger groups may result in a fitness benefit for an individual animal.

One theoretical explanation is that aggregations may form as “selfish herds” (Hamilton 1971), in which an individual reduces its own predation risk at the expense of others in the group. Through this dilution effect, any one individual is less likely to be attacked by a predator due to larger numbers of potential prey. It is also possible to achieve a “cooperative group,” in which an increase in prey density decreases predator success (Milinski 1979). Understanding the functions of animal groups may help us further understand how individuals make the choices they do in response to predation.

Larval amphibians are an excellent system for investigating questions about responses to predation pressure. Tadpoles of many species face the possibility of predation from a variety of sources, and their responses include changes in their

behavior (Skelly and Werner 1990, Skelly 1992, Relyea 2004), life history (Skelly and Werner 1990, Relyea 2007), and morphology (Relyea and Hoverman 2003, Relyea 2004, Schoeppner and Relyea 2008). Many of these antipredator defenses or avoidance behaviors are mediated by chemical cues (Kats et al. 1988, Fraker et al. 2009, Schoeppner and Relyea 2005, Schoeppner and Relyea 2009) which may vary in specificity depending on the prey and predator species involved.

The American toad, *Anaxyrus americanus*, is a species that is particularly well-suited for asking these questions. Due to toxins in the skin it becomes increasingly unpalatable to predators throughout the tadpole stage (Brodie et al. 1978, Formanowicz and Brodie 1982), which may result in them being increasingly protected as they develop and get larger. It also prefers to aggregate with kin in lab and field experiments (Waldman and Adler 1979, Waldman 1982), identifying kin based on chemical cues in the water (Waldman 1985, Waldman 1986). In other species that show similar unpalatability and kin preferences, tadpoles school more cohesively in a single sibship group and in the presence of predator cues (Watt et al. 1997). We might therefore expect that in the American toad a “cooperative group” response to predation might be even more advantageous in a pure sibship group.

If predators must learn that toad tadpoles are unpalatable, aggregating with kin may provide an additional benefit beyond the dilution effects that come from using a group to minimize predation risk. With kin groups, a predator that consumes a tadpole learns that it is distasteful and avoids consuming the original tadpole’s siblings, which tend to share genes by common descent. The idea of inclusive fitness holds that an individual’s overall fitness is more than just its direct genetic contribution to the next

generation (Hamilton 1964, Gardner and West 2014); a tadpole can still increase its inclusive fitness if being consumed by a predator increases the probability that its relatives survive. While predators should learn to avoid tadpoles regardless of the kinship composition of the group, the increased inclusive fitness benefit if the group is composed of relatives could select for kin aggregation in these tadpoles. If predators fail to learn (or learn imperfectly) that these tadpoles are unpalatable, we may still expect to see tadpole aggregation patterns more in line with the selfish herd model.

We designed an experiment to investigate the process underlying how American toad tadpoles respond to predator cues (Table 3.1). By setting up a choice experiment where tadpoles were provided with water free of tadpoles, cues from a group of kin, and cues from a group of non-kin, we hoped to gain an understanding of tadpole aggregation patterns in the presence and absence of predator cues. Under the “selfish herd” model, we expect to see an increased preference for aggregating with tadpoles rather than remaining alone in open water when predator cues are present, with no difference between kin and non-kin. In the “cooperative group” model, given the possibility of predators learning to avoid unpalatable tadpoles and increased inclusive fitness benefit to tadpoles that group with kin, we expect to see a preference for related tadpoles in the presence of predator cues. Larger tadpoles may be at decreased risk of predation (Travis et al. 1985), possibly due to an increase in toxins across development (Brodie et al. 1978), which may lead to weaker aggregation preferences in larger tadpoles. This experiment may help us further our understanding of the patterns of schooling we see in the lab and field and how these aggregations contribute to tadpole fitness.

Table 3.1. Hypotheses for the underlying model governing how tadpoles aggregate in the presence of predators.

H0: Tadpole aggregation behavior does not change in response to predators
H1: Tadpole aggregation in the presence of predators follows the “selfish herd” model
Prediction: Focal tadpoles will be more likely to choose to join groups of other tadpoles, with no preference for kin vs. non-kin, rather than remaining alone in open water
H2: Tadpole aggregation in the presence of predators is kin-selected “cooperative group”
Prediction: Focal tadpoles will be more likely to choose to join groups of kin over non-kin, rather than remaining alone in open water

Methods

We collected pairs of adult American toads in amplexus from several locations in southwest Michigan in May 2014. These locations included the Experimental Pond Laboratory at the Kellogg Biological Station (KBS). Pairs were isolated in buckets between 24 and 48 hours and given a chance to lay eggs, at which point we returned them to their capture site. Nine clutches were laid for use in this experiment. Each clutch was split between two 10-gallon aquarium tanks, so we would later be able to investigate preferences for kin independent of familiarity. Tadpoles were maintained in these tanks until they reached developmental stage 25 as laid out by Gosner (1960), when independent feeding begins.

Our testing apparatus was made up of a plastic storage container 13.5 cm x 35 cm x 50 cm, divided in half by a partition extending three-quarters of the length of the box. Identical 5-gallon buckets provided water to either side via 3/16-inch airline tubing. An outflow hole at the end of the container opposite the buckets allowed for continued water flow during the trials (Fig. 3.1).

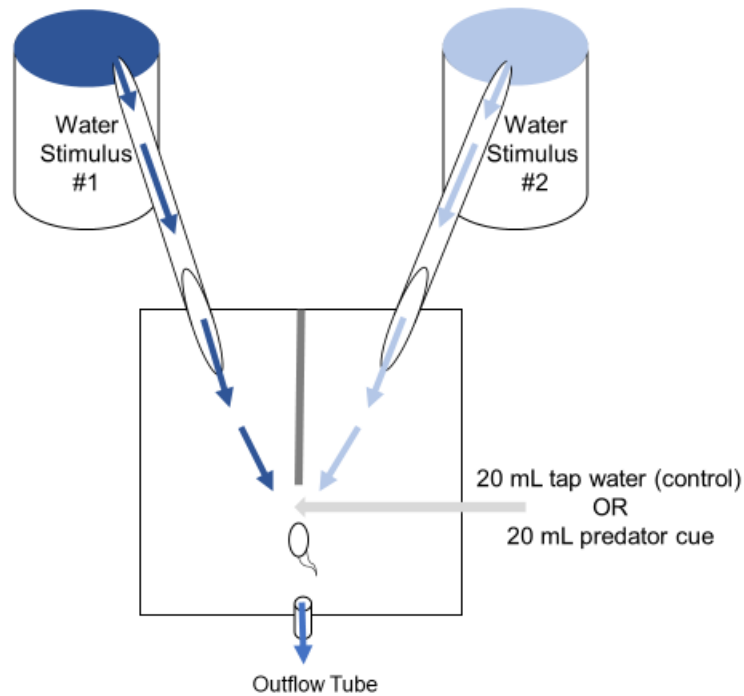


Figure 3.1. Experimental setup for tests of tadpole preference. Two choices were provided in separate buckets, with airline tubing allowing water to flow from the bucket into the testing apparatus. Another piece of airline tubing allowed excess water to flow out the other end of the box. 20 mL of tap water or predator cue were added right before the tadpole was placed in the testing apparatus, and the side the tadpole chose was recorded.

We recorded each tadpole's mass, snout-vent length (SVL), and Gosner stage before beginning preference trials. Tadpoles were provided with three sets of choices in randomized order in each portion of the experiment: kin vs. non-kin, kin vs. control, and non-kin vs. control. The "control" bucket contained only tap water, while other buckets contained water from one of the aquaria containing tadpoles. The water in the "non-kin" stimulus came from an unmixed family of tadpoles other than the one from which the focal tadpole originated, as opposed to a mixture of families. For a given sample of water from a particular tank, we set up the bucket and selected our focal tadpoles from the same family for a "kin" treatment or a different family for the "non-kin" treatment. We

know from previous experiments that water from other tadpoles is sufficient to cause changes in development and behavior.

In this experiment, we used the water bug of the genus *Belostoma* (Hemiptera) as our predator (Swart and Taylor 2004). Predators were collected from ponds at the Experimental Pond Laboratory. We obtained predator cues by placing belostomatid individuals in a container with five tadpoles and using water from this container, which contained any potential odors or cues from the predator itself as well as cues of tadpole consumption, in our trials. Shortly before introducing the focal tadpole to the experiment, we added 20 mL of tap water (in control trials) or 20 mL of a predator cue (in predator trials) to the test area. A tadpole was then added to the testing apparatus and their movement monitored for up to one minute. If a tadpole swam more than two inches up one of the unique sides of the apparatus, we recorded which side they chose and how long it took them. If they did not choose a side before the end of one minute, we recorded “no preference” for that tadpole.

In total, 181 tadpoles were tested in at least one of the treatment combinations. For the kin vs. non-kin choice, 117 tadpoles were tested in both the presence and absence of predator cues, 13 only with predator cues, and 21 only without predator cues. For the kin vs. control choice, 87 were tested in both treatments, 39 only with predator cues, and 48 only without predator cues. For non-kin vs. control, 78 were tested in both treatments, 39 only without predator cues, and 49 only without predator cues.

Statistical Analysis

We used a multinomial logistic regression analysis to determine whether there were significant differences in which choices tadpoles made depending on treatment. We used the *nnet* package (Venables and Ripley 2002) in R (R Core Team 2018) to test whether the presence of predator cues affected the probability of choosing each stimulus. We first fit our response variable (which stimulus each tadpole chose, or whether it showed no preference) to predator treatment for each of the three comparisons (kin vs. non-kin, kin vs. control, and non-kin vs. control). We also fit a version of the models including an interaction with focal tadpole body mass and predator treatment. We generated p-values using the Wald chi-square test.

Results

We asked whether the addition of predator cues to our experimental system altered the probability that focal tadpoles would choose one stimulus over the other in three sets of comparisons: kin vs. non-kin, tap water vs. non-kin, and tap water vs. kin. In all three comparisons, we saw that tadpoles were significantly less likely to show no preference compared to choosing one side over the other (Kin vs. Non-Kin: $z = -3.51$, $p = 0.0004$; Control vs. Non-Kin: $z = -3.67$, $p = 0.0002$; Control vs. Kin: $z = -2.55$, $p = 0.011$), and this did not change with the addition of predator cues (Kin vs. Non-Kin: $z = -0.54$, $p = 0.59$; Control vs. Non-Kin: $z = -0.23$, $p = 0.82$; Control vs. Kin: $z = -0.27$, $p = 0.79$).

While we saw trends in the probability that tadpoles exhibited certain preferences, we observed high variation and few trends were significant. When

presented with a choice between water from kin and water from non-kin in the absence of a predator cue, tadpoles were slightly more likely to choose non-kin (Fig. 3.2A, $z = 0.47$, $p = 0.64$), but were more likely to choose kin when exposed to a predator cue (Fig. 3.2A, $z = -0.73$, $p = 0.46$). Tadpoles chose a control stimulus of plain water more frequently than non-kin under control conditions (Fig. 3.3A, $z = -0.98$, $p = 0.33$), but the presence of a predator cue increased the probability of choosing non-kin (Fig. 3.3A, $z = 1.26$, $p = 0.21$). Tadpoles chose kin over plain water in both control (Fig. 3.4A, $z = 0.48$, $p = 0.63$) and predator (Fig. 3.4A, $z = 0.78$, $p = 0.44$) conditions.

When considering mass as well as presence of predator cues in our model, we saw additional trends. In the choice between kin and non-kin in control conditions, tadpoles were more likely not to exhibit a preference as their mass increased (Fig. 3.2B, $z = 1.32$, $p = 0.19$), while in the predator treatment larger tadpoles were more likely to associate with kin than with non-kin or exhibit no preference (Fig. 3.2B). We saw a similar pattern where larger tadpoles were more likely not to have a preference between non-kin and tap water in the control treatment (Fig. 3.3B, $z = 1.10$, $p = 0.27$), although with this choice we saw that larger tadpoles were more likely to choose tap water over unrelated conspecifics (Fig. 3.3B). In the final set of comparisons, we again saw larger tadpoles being more likely to exhibit no preference between kin and tap water in the control treatment (Fig. 3.4B, $z = 0.59$, $p = 0.56$). In the predator cue treatment, the probability of choosing kin remained constant across mass (Fig. 3.4B, $z = -0.38$, $p = 0.71$), while the likelihood of choosing tap water increased as mass did (Fig. 3.4B). Although none of these trends were significant, some of the patterns suggest interesting dynamics which we discuss below.

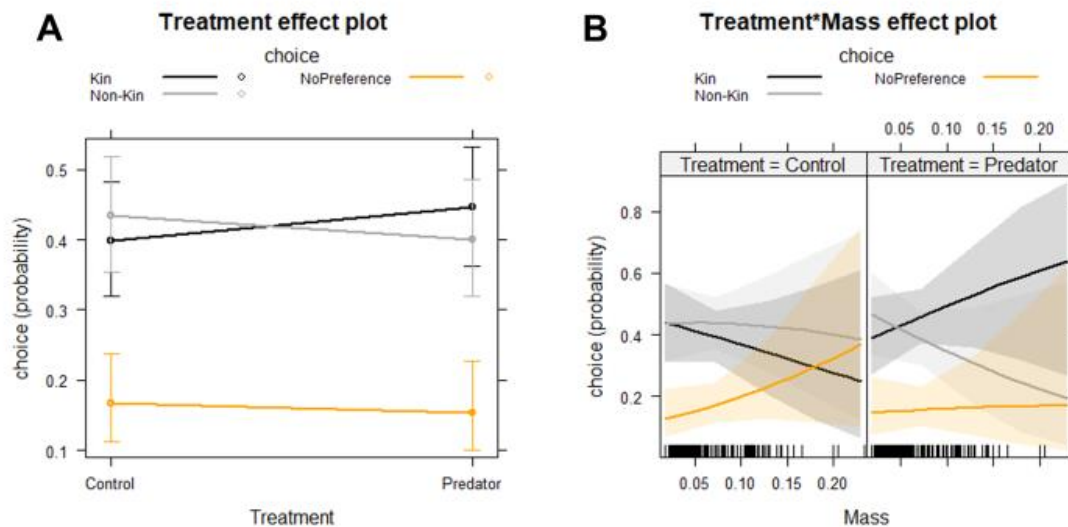


Figure 3.2. Relationship between the probability that the focal tadpole chose kin, non-kin, or showed no preference and (A) the presence or absence of predator cues and (B) the interaction between the presence or absence of predator cues and focal tadpole mass (in grams). Black indicates kin, gray indicates non-kin, and orange indicates no preference. Error is $\pm$ SE.

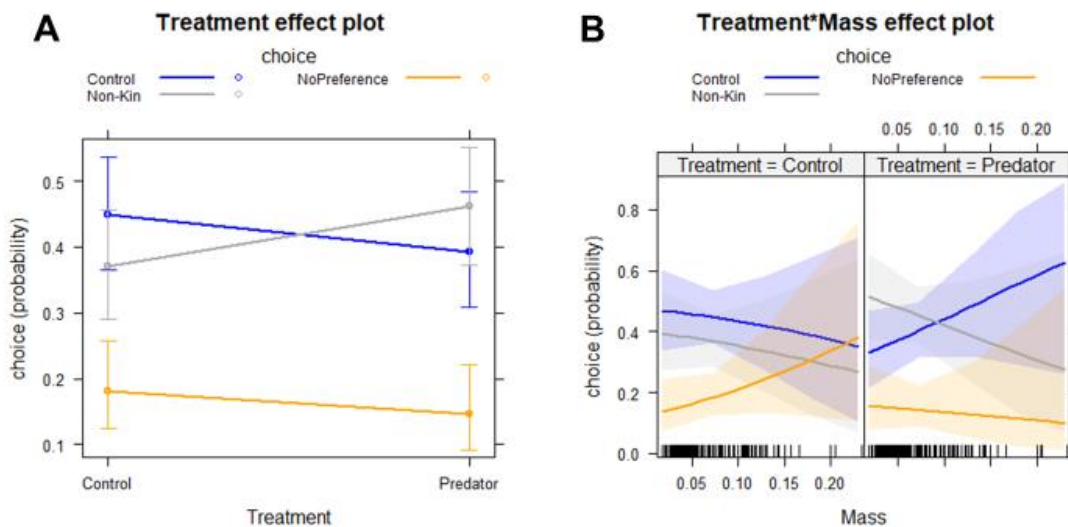


Figure 3.3. Relationship between the probability that the focal tadpole chose control tap water, non-kin, or showed no preference and (A) the presence or absence of predator cues and (B) the interaction between the presence or absence of predator cues and focal tadpole mass (in grams). Blue indicates control tap water, gray indicates non-kin, and orange indicates no preference. Error is $\pm$ SE.

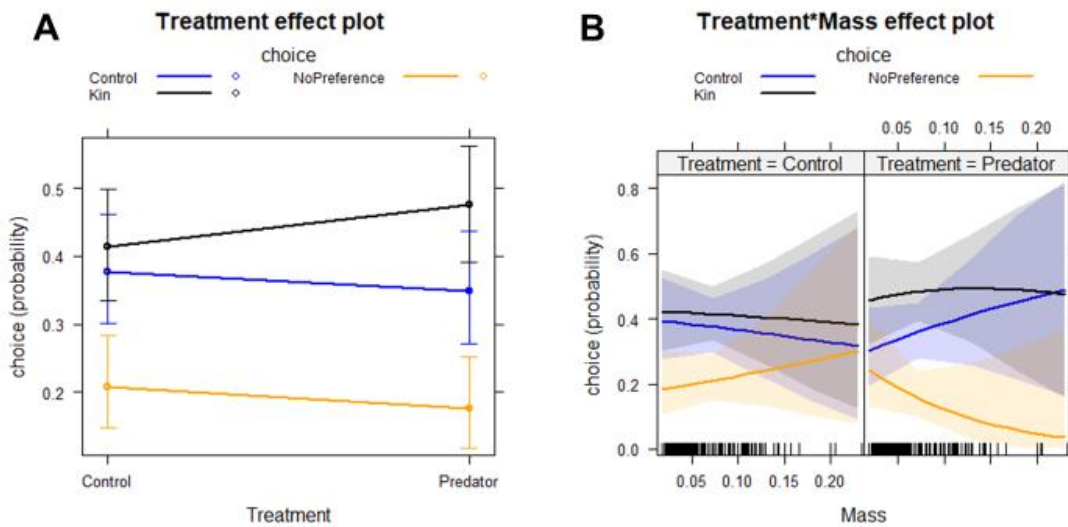


Figure 3.4. Relationship between the probability that the focal tadpole chose control tap water, kin, or showed no preference and (A) the presence or absence of predator cues and (B) the interaction between the presence or absence of predator cues and focal tadpole mass (in grams). Blue indicates control tap water, black indicates kin, and orange indicates no preference. Error is $\pm$ SE.

Discussion

We found that tadpoles in our experiment were more likely to choose one stimulus over the other than remaining in their starting position, regardless of whether predator cues were present (Fig. 3.2A, Fig. 3.3A, Fig. 3.4A). As most of our other results were not statistically significant, we were unable to find support for either of our hypotheses governing how tadpoles might respond to the presence of predators (Table 3.1). The results we did see show several interesting trends that suggest relatedness to a group may influence how tadpoles respond to predators if these patterns are indeed reflective of the underlying processes.

There are several possible reasons why we were unable to detect significant differences in preference between treatments. The simplest possibility is that we were unable to capture significant patterns because those patterns do not exist. Our

experimental setup may have failed to capture the ways tadpoles behave in response to predators, or it may be that tadpoles do not use the types of chemical cues we provided to inform their antipredator responses. Our previous work has shown that differences in chemical cues are sufficient to cause differences in tadpole growth, but chemical cues alone may not exactly reproduce patterns seen when tadpoles may also interact physically with one another (previous chapters). While it is likely that our experimental setup provided tadpoles with some relevant information in the water, there may be other aspects of their environment or social interactions necessary for antipredator responses in the field. The location of the outflow tube (Fig. 3.1) may have also weakened the strength of predator cues the tadpoles received.

Even if our focal tadpoles were able to distinguish between kin and non-kin given our stimuli, they may require stronger antipredator cues to shape their responses. In this experiment, the focal tadpole could potentially identify the presence of a predator, but all stimulus water came from tanks where the tadpoles were not experiencing predator cues. In previous work, *Bufo bufo* tadpoles schooled more cohesively when with kin in the presence of predator cues (Watt et al. 1997). Tadpoles may choose which group to swim toward in response to a predator cue, but there may be stronger pressure to do so if the groups are also exhibiting a predator response. The rest of the tadpoles in the group may also need to be exposed to evidence of a predator to result in the most advantageous collective response.

It is also possible that the identity of the predator will influence tadpole response. The presence of toxins in the skin of *Anaxyrus americanus* tadpoles renders them unpalatable to many common predators (Brodie et al. 1978, Formanowicz and Brodie

1982). If a predator must learn to avoid toad tadpoles through experience, schooling with kin would be beneficial from an inclusive fitness perspective. If the predator tries to eat a tadpole and learns to avoid other toad tadpoles (the first tadpole's kin) as a result, there is still an inclusive fitness benefit to the predated tadpole. The water bug pierces the body of its prey and consumes the insides, bypassing the toxic skin altogether; it has been suggested that predators that feed in this way may not find tadpoles unpalatable, unlike those that bite or taste their prey, such as many vertebrate predators (Peterson and Blaustein 1992, Jara and Perotti 2009). If tadpoles are capable of distinguishing between different types of predators, the water bug would not impose the same selective pressure toward kin aggregation. It is unclear whether tadpoles are capable of that fine-scale predator discrimination or whether they simply respond to cues of conspecific stress, but, if so, it may result in the lack of significance we see in this experiment.

We did see several patterns that suggest it could be advantageous to school with other tadpoles, particularly kin. Given a choice between tap water and non-kin, tadpoles were more likely to swim toward the tap water under the control scenario with a switch to preferring non-kin in the presence of predator cues. Tadpoles were more likely to choose kin over tap water in both scenarios, but the likelihood of making that choice increased in the predator treatment. When given a choice between kin and non-kin, tadpoles were slightly more likely to choose non-kin in the control but preferred kin when exposed to predator cues. Together, these results, while not statistically significant, suggest that there is some benefit to grouping with other tadpoles when predators are present, and choosing kin is preferable when they are available.

In addition to these results, we also saw some potentially interesting (although not statistically significant) patterns when looking at how tadpole body mass influenced the probability of choosing each option. In all control scenarios, the probability that a focal tadpole did not choose a side increased with body mass. In the predator cue treatments, we saw that increased body mass correlated with increased probability of choosing tap water while probability of choosing non-kin declined. Probability of choosing kin over tap water was fairly constant across body mass, while increasing body mass actually correlated with increased likelihood of choosing kin over non-kin. In general, this suggests that larger tadpoles, who may be at decreased risk of predation from at least some predators (Travis et al. 1985), do not receive as great a benefit from schooling with conspecifics in response to predators, but we still saw larger individuals choosing kin. If this reflects an underlying pattern, this suggests that there may be additional benefits to aggregating with kin beyond those outlined in the “selfish herd” hypothesis (Hamilton 1971).

Predation is likely not the most significant selective pressure driving the tendency for toad tadpoles to associate with kin, but it has the potential to be part of the story. Individuals derive a direct benefit from forming groups in response to predation pressure, as it reduces the likelihood that any individual is chosen, but there are additional aspects of American toad biology that may provide benefits for choosing kin over non-kin. To better test our hypotheses in future experiments, it would be beneficial to expose stimulus tadpoles to predator cues as well as testing whether the type of predator alters tadpole response. The physical complexity of the testing environment could also prove important, as it may provide alternate refuge for tadpoles in dangerous

situations. If the patterns we saw hold true, it is not enough to know whether an individual is exposed to predation to predict its prospects for survival. Understanding how relatedness modifies the effects of various selective pressures on fitness is necessary for a more complete understanding of how individuals navigate their physical and social environment and make choices influencing their behavior and survival.

CONCLUSION

I found that tadpole growth rates differed in response to cues of resource and kinship environment. For tadpoles that did not reach metamorphosis, the combination of kinship and resource cues was important in predicting growth rates. Tadpoles receiving cues from kin in an environment with abundant resources showed evidence consistent with deferral. In another experiment, tadpole growth rate differed based on cues of relative size, with larger tadpoles outperforming smaller partners. This relationship was affected by kinship, at least for smaller tadpoles, who grew more rapidly if their partner was a sibling. In both experiments, tadpoles were equally likely to metamorphose across treatments when receiving cues from kin, while proportion surviving to metamorphosis was lower in some treatments receiving cues from non-kin. This indicates that chemical cues communicate information necessary for tadpoles to perceive aspects of their environment, which interact with relatedness to affect fitness.

In a cattle tank experiment, I found strong evidence that kinship affects aspects of development that are likely important for fitness in American toad tadpoles and that these effects were context-dependent. Tadpoles grew more over the course of the experiment when grouped with siblings, but the greatest differences between kinship treatments were seen under conditions where competition had the potential to be more intense. Comparative benefits of kinship disappeared in nutrient enriched treatments, when cooperating with kin might not provide enough of an additional fitness benefit to select for such strategies.

In the final experiment, I saw that tadpoles given a choice between two stimuli (two of tap water, kin cues, and non-kin cues) were more likely to choose one side over the other rather than showing no preference. In the absence of predator cues, we saw that tadpoles may be more likely to choose tap water over non-kin, kin over tap water, and non-kin over kin. Increasing body mass also likely increased the probability of exhibiting no preference. In the presence of predator cues, tadpoles may be more likely to choose non-kin over tap water, continue to prefer kin over tap water, and choose kin over non-kin. While increased body mass might result in a tadpole being more likely to choose tap water in the presence of predator cues, we saw that larger tadpoles potentially increased the probability of choosing kin over non-kin. While many of these predator cue results were not significant, indicating that predator avoidance is likely not the primary driver of kin aggregation behavior in this species, they are suggestive of a kin-selected benefit to grouping beyond the selfish herd.

Tadpoles grow and develop differently in response to variation in environmental conditions; these experiments indicate that their perceptions of their environment are influenced by chemical cues in the water. Physical interactions with conspecifics and the surrounding habitat are necessary for understanding the full picture, but we have seen that exposure simply to water from different conditions communicates information about relatedness and relative size of conspecifics as well as resource availability.

These differences influence the proportion of tadpoles surviving to metamorphosis as well as altering how quickly they get there, both of which affect fitness. Responding to variation in the environment requires tadpoles to be able to perceive this variation; understanding the role of chemical cues allows us to make

sense of how variation in more complex ecological contexts results in changes in fitness.

The way kinship modifies the effects of environmental conditions on fitness likely operates not only in American toads, but also in other species that can interact with relatives and non-relatives during development. We suggest that further studies that isolate, and manipulate, both relatedness and ecological contexts will offer powerful insights to the evolution of kin recognition, cooperation, and their modulation of inclusive fitness. Our findings confirm the importance of considering how environmental context influences the subtle costs and benefits of competing or cooperating with kin. They suggest that complex interactions between population structure and local environments are likely to shape the evolution of both kin discrimination, and the ways it is used to maximize inclusive fitness. Considering these factors in isolation is likely to lead to an incomplete understanding of the natural world, and these results thus represent an important contribution to enhanced understanding of how populations evolve in different contexts.

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